CURRENT ISSUE

British Journal of Pharmacology

NO

September 1975

3

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The British Journal of Pharmacology is published monthly by Macmillan Journals Ltd, 4 Little Essex Street, London WC2R 3LF. Telephone: 01-836 6633.

Annual subscription £36.00 ($90 USA and Canada). (Orders must be accompanied by remittance. Prices applicable only to orders received before 31st December 1975.)

Cheques should be made payable to Macmillan Journals Limited: Subscription Department, Brunel Road, Basingstoke, Hampshire RG21 2XS, England.

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Second class postage paid at New York, N.Y.
US Mailing Agent, Air and Sea Freight Inc., 527 Madison Avenue, New York, NY 10022.

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CT **Chemotherapy**
M **Miscellaneous**

PM **Pharmacometrics**
PP **Psychopharmacology**
TX **Toxicology**

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PRESSOR RESPONSE INDUCED BY LOCAL ANAESTHETICS PERFUSED THROUGH THE CEREBRAL VENTRICLES OF DOGS

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1 The antihistamines mepyramine and halopyramine which have local anaesthetic properties, produced a rise in arterial blood pressure followed by a fall, similar to that seen with procaine, when perfused in a 10 mg/ml solution from the lateral ventricle to the cisterna magna in dogs.

2 The pressor response produced by procaine perfused through the cerebral ventricle is due to an action on structures situated in the caudal half of the floor of the fourth ventricle because a pressor response was obtained when a few microlitres of a 50 mg/ml solution of procaine were applied to a small area of the exposed floor of the fourth ventricle in a region 2 to 4 mm rostral to obex, and after cauterization of this area procaine perfused through the cerebral ventricles no longer raised arterial blood pressure.

3 Bilateral denervation of the carotid sinus did not affect the pressor response obtained with procaine perfused through the cerebral ventricles.

4 The pressor response to procaine is mediated through the sympathetic nervous system. It results partly from an increased adrenaline discharge from the adrenal glands and partly from increased sympathetic vasomotor tone, because the response is abolished or attenuated after removal of the adrenal glands and intravenous injections of hexamethonium and phentolamine.

Introduction

Procaine, injected or perfused into the cerebral ventricles of unanaesthetized dogs or anaesthetized dogs and cats, was shown to produce a biphasic blood pressure response, i.e. an initial rise followed by a fall in arterial blood pressure (Haranath, Naseem-Ayesha-Begum & Sitaramayya, 1965; Haranath & Venkatakrishna-Bhatt, 1968a; Borison, Haranath & McCarthy, 1972). Similar results were obtained with two other local anaesthetics, cinchocaine and lidocaine (Haranath & Venkatakrishna-Bhatt, 1968b), as well as with propranolol which also has local anaesthetic properties. The results with propranolol were obtained on its injection into the cerebral ventricles of anaesthetized dogs (Shrivastava, Kulshrestha, Singh & Bhargava, 1973) and of unanaesthetized rabbits (Dollery, Lewis, Myers & Reid, 1973) and cats (Day & Roach, 1974).

An initial rise followed by a fall would thus appear to be the characteristic blood pressure response to local anaesthetics when they are injected into or perfused through the cerebral ventricles. This suggestion is supported by the present experiments in which the same response was obtained with two antihistamines, mepyramine and halopyramine, that have local anaesthetic properties.

Further experiments deal with the site at which procaine acts when producing its pressor effect. Previous experiments suggested structures in the distal half of the floor of the fourth ventricle as the site because the response occurred on perfusion of the cerebral ventricles when the procaine was prevented from entering the lateral ventricles, the third and the rostral half of the fourth ventricle (Haranath *et al.*, 1965) whereas no pressor response was obtained when the procaine was perfused through the subarachnoid space surrounding the brain stem (Haranath & Venkatakrishna-Bhatt, 1968a). The procedure adopted in the present experiments for localizing the site of action in the distal half of the floor of the fourth ventricle was to apply the procaine topically to small areas of the exposed floor and to show that its intraventricular injection no longer produced a rise in arterial blood pressure when the area from which a pressor response had been obtained on topical application was cauterized. Finally, in order to find out if the pressor response was due to blockade of impulses from the carotid sinus nerves and if it resulted from activation of the sympathetic nervous system, the effects of denervation of the carotid sinus, of removal of the suprarenals and of intravenous injections of hexamethonium and phentolamine on the pressor response were examined.

Methods

Dogs weighing 5 5 to 15 kg were used. They were anaesthetized with intravenous chloralose (100 mg/kg). The blood pressure was recorded from the cannulated right femoral artery with a mercury manometer, and the drugs were injected into the cannulated right femoral vein. The trachea was cannulated so that artifical ventilation could be applied when necessary.

For the perfusion of the cerebral ventricles, from the left lateral ventricle to the cisterna magna, the method used was that described for cats by Bhattacharya & Feldberg (1958). The rate of perfusion was 0.1 ml/minute.

For the topical application of procaine to small areas of the distal half of the floor of the fourth ventricle and for cauterization of these areas the cisterna magna was opened by cutting away the exposed atlanto-occipital membrane and enlarging the opening by nibbling away the occipital bone which forms the rostral margin of the cisterna. The cerebellum was then elevated with a spatula. The procaine solution (50 mg/ml) was applied with a fine polyethylene tube PE 10 attached to a AGLA brand micrometer syringe (B.W.Co) which is capable of delivering fractions of a microlitre. Gentle cauterization was used to produce discrete lesions in different parts of the distal half of the floor of the fourth ventricle and the side of the medulla. A thermal cautery heated to turn cotton wool brown on contact was used for this purpose. In one experiment the area postrema was cauterized on both sides under aseptic conditions. The success of the ablation was tested functionally seven days later by intravenous apomorphine which no longer produced emesis. The dog was then anaesthetized with chloralose and the cerebral ventricles were perfused with procaine.

To denervate the carotid sinus, the tissue behind the bifurcation of the carotid artery, excluding the internal and external carotid artery, was cut between two mass ligatures. To remove the adrenal glands the paravertebral retroperitoneal approach was used.

Drugs

The following drugs were used: procaine hydrochloride (May & Baker), mepyramine maleate (May & Baker) (the concentrations given for these drugs refer to their salts), halopyramine (Synopen of Suhrid Geigy) and phentolamine (Regitine, Ciba). The composition of the artificial cerebrospinal fluid (c.s.f.) used for perfusion of the cerebral ventricles and the method of preparing the isotonic solution of procaine hydrochloride were the same as described previously (Haranath *et al.*, 1965).

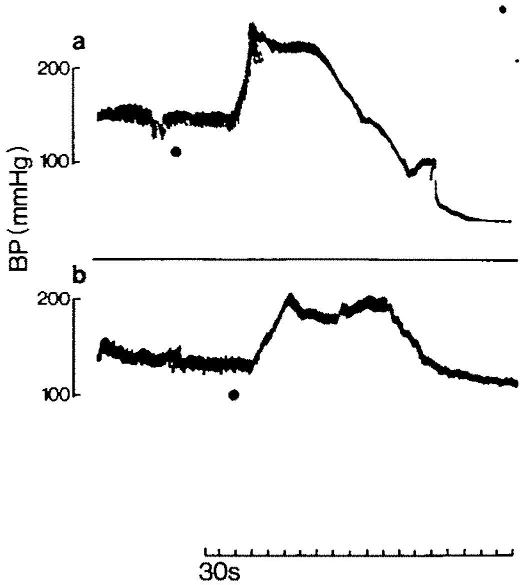

Figure 1 Records of arterial blood pressure obtained from 2 dogs (a and b) under chloralose anaesthesia. From the black dot, in (a) mepyramine maleate (10 mg/ml) and in (b) halopyramine (10 mg/ml) were continuously perfused from the lateral ventricle to the cisterna magna until the end of the experiment.

Results

Experiments with mepyramine and halopyramine

Perfusion of the cerebral ventricles, from the lateral ventricle to the cisterna magna, with a 10 mg/ml solution of either mepyramine or halopyramine resulted in a rise of arterial blood pressure followed by a fall. This is illustrated by the two experiments (a) and (b) of Figure 1. In (a) blood pressure began to rise about 2 min after the onset of perfusion with mepyramine, rose from about 150 mm to over 200 mmHg, remained elevated for less than 3 min and then fell to below 40 mmHg. In (b) blood pressure began to rise within 1 min of the onset of perfusion with halopyramine, rose from about 120 mm to about 200 mmHg, remained high for about 3 min and progressively declined although the perfusion with halopyramine continued (not included in the figure). In all the experiments, three with mepyramine and two with halopyramine, the

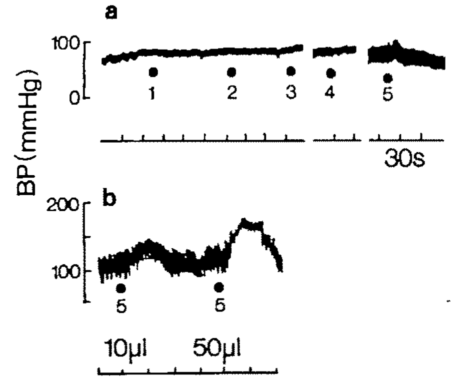

Figure 2 Records of arterial blood pressure obtained from 2 dogs (a and b) under chloralose anaesthesia with topical application of procaine to the floor of the fourth ventricle and adjoining areas. In (a) 2 µl of procaine (50 mg/ml) was applied to either side of medulla (1 and 3), 2-4 mm caudal to the obex (2), at the obex (4) and 2-4 mm rostral to it (5). In (b), 10 and 50 µl of procaine (50 mg/ml) were applied 2-4 mm rostral to the obex. This area is indicated in the inset of Figure 3.

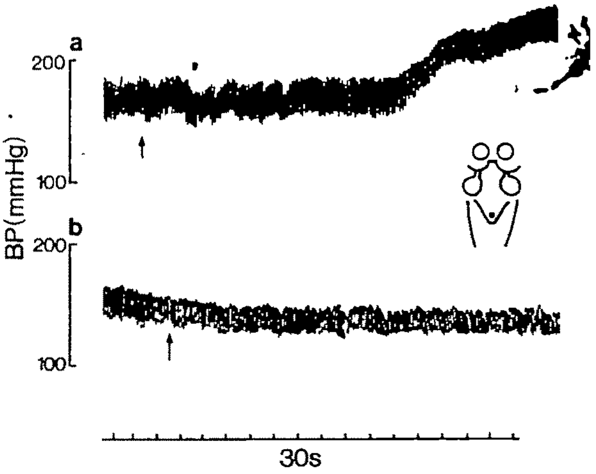

Figure 3 Record of arterial blood pressure obtained from a dog under chloralose anaesthesia. The lower record was obtained after cautery of an area on the floor of fourth ventricle a little rostral to the obex. The area is indicated in the inset. Arrows indicate start of procaine perfusion (20 mg/ml) at 0.1 ml/min from the lateral ventricle to the cisterna magna.

pressor response was followed by a fall below the preperfusion level.

Experiments with procaine

Site of action In four experiments 2 µl of a 50 mg/ml solution of procaine was applied to the sides of the medulla oblongata, or to the obex or to various other areas of the caudal half of the floor of the fourth ventricle. As shown in Figure 2 no blood pressure effects were obtained with this procedure except when the procaine was applied to an area 2 to 4 mm rostral to the obex. Then its application elicited a small rise in arterial blood pressure, and when this area was flooded with 10 or 50 µl of the procaine solution a strong pressor response ensued (Figure 2b). There was no area from which a depressor response was obtained on topical application of procaine to the rostral half of the floor of the fourth ventricle.

In eight experiments lesions of about 2 to 2.5 mm in diameter were made by cauterization in the area rostral to the obex which corresponded to the area from which procaine elicited a pressor response on topical application. After cauterization of this area perfusion of the cerebral ventricles with a 20 mg/ml solution of procaine no longer produced a pressor response in six, and produced a greatly diminished response in two experiments. The two records of Figure 3 illustrate one of the experiments in which the pressor response was abolished. Record (a) was obtained before cauterization; it shows the usual rise in arterial blood pressure which began within 6 min of the onset of the procaine perfusion. Record (b) was obtained after cauterization and the procaine perfusion no longer raised the arterial blood pressure. When similar lesions were made more rostrally, yet still within the caudal half of the floor of the fourth ventricle the pressor response persisted on subsequent perfusion of procaine through the cerebral ventricles. In one experiment in which the area postrema had been cauterized in an aseptic operation, perfusion of the cerebral ventricles with a 20 mg/ml procaine solution seven days later, produced the normal response followed by a fall in arterial blood pressure.

Carotid sinus denervation Bilateral denervation did not abolish the pressor response obtained when procaine was perfused in a 20 mg/ml solution through the cerebral ventricles. This result was obtained in six experiments, one of which is illustrated in Figure 4. Record (a) was obtained before denervation and shows the usual pressor responses to bilateral occlusion of the carotid arteries and the rise in blood pressure which began within 2 min of the onset of the procaine perfusion. Record (b) was obtained from the same dog after denervation. The success of the denervation was evident from the fact that the bilateral occlusion of the carotid arteries no longer produced pressor responses. Yet on perfusion of

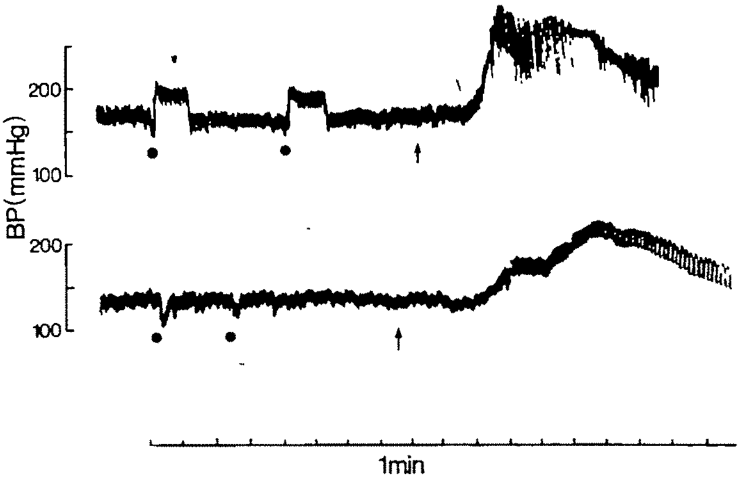

Figure 4 Record of arterial blood pressure obtained from a dog under chloralose anaesthesia. The lower tracing was taken after carotid denervation. At the black dots, bilateral carotid occlusion for 1 minute. At the arrows, perfusion of procaine (20 mg/ml) started from the lateral ventricle to the cisterna magna at 0.1 ml/minute.

the cerebral ventricles with procaine the blood pressure began to rise within 3 minutes.

Adrenalectomy In three dogs in which the perfusion of the cerebral ventricles with procaine (20 mg/ml) had produced strong pressor responses, the adrenal glands were removed on both sides. This itself lowered the blood pressure by 0 to 30 mmHg and in two it abolished the pressor reponse to perfusion of the cerebral ventricles in the procaine; in the third a much attenuated pressor response was obtained.

Hexamethonium and phentolamine In five experiments hexamethonium (4.5 to 6.3 mg/kg) was injected intravenously. This caused a fall in arterial blood pressure of 40 to 60 mmHg and abolished the pressor response to perfusion of procaine (20 mg/ml) through the cerebral ventricles. In two experiments phentolamine (6.7 and 10.8 mg/kg) was injected intravenously. This attenuated but did not abolish the pressor response to the perfusion with procaine.

Discussion

The present experiments support the view that procaine, cinchocaine and lidocaine have pressor actions when injected into the cerebral ventricles because they are local anaesthetics. Other substances that have local anaesthetic properties should therefore have similar actions, even if they have other pharmacological properties for which they are used. Propranolol, for instance is used clinically for its β-adrenoceptor blocking action, but in addition it has local anaesthetic properties and it was found to produce a pressor effect when injected into the cerebral ventricles (Shrivastava *et al.*, 1973; Dollery *et al.*, 1973). Mepyramine and halopyramine are antihistamines, which have relatively strong local anaesthetic properties as well, and in the present experiments they were shown to produce a rise in arterial blood pressure when perfused through the cerebral ventricles.

It was previously concluded that the pressor response to procaine perfused through the cerebral ventricles was due to an action on structures situated in the caudal part of the floor of the fourth ventricle, since it was obtained when the other parts of the ventricular system were excluded from the perfusion with procaine. This conclusion is supported by the results of the present experiments in which the procaine was applied topically to the floor of the fourth ventricle or perfused through the cerebral ventricles after having made lesions to the floor of this ventricle. With these methods the site where procaine acts when producing its pressor effect could be pinpointed to a 5 mm^2 area in the caudal half of the floor of the fourth ventricle 2 to 4 mm rostral to the obex. This area corresponds to the well known depressor or vasodilator area from which a fall in arterial blood pressure is obtained on electrical stimulation (Alexander, 1946; Lindgren & Uvnas, 1954) and which is also stimulated by nicotine (Guertzenstein, 1971). It can therefore be concluded that procaine produces a rise in arterial blood pressure because it removes the inhibitory tone exerted from this area.

It has always been assumed that the inhibitory tone from this area is mediated through the sympathetic nervous system. Results of the present experiments support this view as the pressor response to procaine was abolished or attenuated by procedures which interfere with the sympathetic discharge, i.e. removal of the adrenal glands, intravenous hexamethonium and phentolamine. The relative importance of increased adrenaline discharge from the adrenal glands and of increased vasoconstrictor tone in the pressor response to procaine seems to vary, because in three experiments the pressor response was abolished twice and attenuated once by removal of the adrenal glands.

The present experiments do not deal with the late depressor effect following the initial pressor effect on perfusion of cerebral ventricles with procaine. The depressor response to application of procaine to cerebral ventricles in anaesthetized dogs was described by Loeschcke & Koepchen (1958). Mitchell, Loeschcke, Massion & Severinghaus (1963) and Severinghaus, Mitchell, Richardson & Singer (1963) suggest a chemoreceptor area in the ventral surface of the brain stem as the site of action of this depressor effect. Haranath & Venkatakrishna-Bhatt (1958a) observed only a fall in blood pressure when procaine was injected into the subarachnoid space around the brain stem through an open cisterna, while at the same time its access to the fourth ventricle was prevented by a simultaneous perfusion from the lateral ventricle with artifical c.s.f. at twice the speed.

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(Received May 30, 1974.
Revised February 1, 1975.)

Br. J. Pharmac. (1975), 55, 9-15

TEMPERATURE AND BEHAVIOURAL RESPONSES INDUCED IN THE UNANAESTHETIZED CAT BY THE CENTRAL ADMINISTRATION OF RX72601, A NEW ANTICHOLINESTERASE

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1 The central effects produced by a new anticholinesterase RX72601 injected into the cerebral ventricles or perfused within circumscribed forebrain structures have been investigated in the unanaesthetized cat.

2 When injected into the cerebral ventricles in doses of 1-10 μg, RX72601 produced a dose-related hyperthermia together with behavioural and autonomic responses.

3 Experiments designed to localize the site or sites of these actions demonstrated that hyperthermia could be produced from several diencephalic sites but that the other effects were not normally produced from these same sites.

Introduction

Two distinct types of evidence support the concept that a cholinergic pathway within the hypothalamus mediates heat production (Myers, 1971a). Firstly, acetylcholine (ACh) and cholinomimetic substances injected into the ventricles or directly into the brain substance produce a rise in body temperature in a number of species (for review, see Myers, 1974). Secondly, on perfusion of discrete hypothalamic sites in the monkey with push-pull cannulae, it has been shown that the release of ACh from such sites is increased mainly by cooling and suppressed by warming the primate's body (Myers & Waller, 1973).

Attempts to identify the type of cholinoceptor involved in thermoregulatory processes have also proceeded along two separate avenues. By means of micro-injection techniques in primates, Myers and his co-workers have mapped out areas of the diencephalon most sensitive to cholinergic stimulation using carbachol (Myers & Yaksh, 1969) and later nicotine (Hall & Myers, 1971). Briefly, these workers found that whilst both drugs produced hyperthermia when injected into the posterior hypothalamus only carbachol did so when injected into the anterior hypothalamus and preoptic area. A similar study was undertaken in the cat by Rudy & Wolf (1972) who also used carbachol as their cholinomimetic agent. They too found that this substance was capable of producing hyperthermia in both the anterior and posterior regions of the hypothalamus. Although the drug-induced changes in body temperature reported by all of these workers depend on the site of injection and the dose administered, it is not clear whether the receptors involved can be clearly designated muscarinic or nicotinic as is the custom for peripheral cholinoceptors.

The second approach was pharmacological rather than anatomical in nature. Hall (1972) induced hyperthermia in cats by the intraventricular injection of carbachol. Similar injections of nicotine produced hypothermia. The carbachol-induced hyperthermia was abolished by atropine but not by hexamethonium or mecamylamine. Conversely, the nicotine hypothermia was blocked by hexamethonium or mecamylamine but not by atropine. Although these findings support the view that cholinergic hyperthermia results from stimulation of 'muscarinic' receptors whilst cholinergic hypothermia is produced as the result of stimulating 'nicotinic' receptors, nicotine injected directly into the caudal hypothalamus of the monkey produces a rise in temperature (Hall & Myers, 1972).

All the experiments described so far have sought to identify and characterize responses achieved by the direct introduction of cholinomimetic agonists into the central nervous system (CNS). An alternative approach is to observe the effects produced when the breakdown of endogenous ACh is prevented at selected CNS sites by the prior administration of an anticholinesterase to those sites. This paper reports the effects observed when the anticholinesterase RX72601, i.e. cis-2-(3-hydroxy-phenyl)-1-pyrrolidino cyclohexane methobromide (Dettmar, Lewis, Metcalf, Readhead, Smith, Gillett, Hedges & Richens, 1974a; Dettmar, Metcalf, Smith, Gillett, Hedges & Richens, 1974b) was injected into the ventricles, or perfused at selected diencephalic sites, in the conscious cat. This compound is one of a series of anticholinesterases recently developed (Lewis, Readhead, Metcalf, Smith & McNally, 1973) and was considered suitable for the present study because it has been shown to lack direct agonist activity at post-synaptic cholinoceptor sites (C.F.C. Smith, unpublished data).

Methods

Seventeen cats of either sex initially in the weight range of 2.6-3.5 kg were used. At least 48 h were allowed to elapse between subsequent experiments on individual animals.

Surgical procedures

Using the procedures described by Myers (1972) an array of either two or four guide tubes was implanted in each animal. Each guide tube consisted of an appropriate length of 17 gauge stainless steel tubing fitted with an indwelling stylet of the same length. The stereotaxic co-ordinates were chosen so that in animals implanted with two bilateral guide tubes the tips rested above the lateral ventricles, whilst in animals implanted with four guide tubes the bilateral arrangement allowed the tips to rest 4-6 mm above the rostral and caudal hypothalamus on each side of the brain. After the cannula array had been lowered to the appropriate position the guide tubes were anchored to the calvarium with cranioplast cement and stainless steel screws. A screw-top polyethylene pedestal was fastened over the array to protect the cannulae and prevent subsequent infection.

Ventricular injections

An injection was made into either lateral ventricle by means of a 20 gauge stainless steel needle which was lowered through the guide tube until contact with the ventricles was established (Myers, 1971b). At this time the solution to be injected passed rapidly into the ventricle by gravity flow. With the exception of one series of experiments, a standard volume of 0.2 ml was used for all intraventricular injections.

Push-pull perfusions

To perfuse a discrete region of the diencephalon push-pull cannulae were used as described previously (Myers, 1970). The outer or pull cannula was constructed from 20 gauge stainless steel thin-wall tubing (Popper Co., New York, N.Y.) and the inner or push cannula from 27 gauge tubing. The push cannula was positioned so that its tip extended 0.8-1.0 mm beyond the end of the pull cannula. This concentric push-pull assembly was lowered through a guide tube to a depth controlled by a polyethylene spacer which fitted tightly over the shaft of the pull cannula. Both the push and pull cannulae were connected by polyethylene tubing to a 2.0 ml calibrated glass syringe (Becton, Dickenson & Co.) mounted on a 4-channel infusion-withdrawal pump (Harvard Apparatus Co.). All perfusions were made at the rate of 50 μl/minute.

Injection solutions

All solutions were prepared with glass distilled ion-exchanged water. An artificial cerebrospinal fluid (CSF) (Myers, 1971b) containing the chloride salts of the following cations (mM) Na^+ 145.0, Ca^{2+} 1.3, K^+ 3.5, and Mg^{2+} 1.0 was used as the vehicle for drug injections. Both RX72601 and neostigmine (Sigma Ltd) were used as the bromide salts; doses refer to the salts. RX72601 (mol wt. 340) was supplied by Reckitt & Colman Ltd, Hull. Each solution was rendered pyrogen-free by standard autoclaving procedures, and in addition was passed through a 0.22 μm Swinnex Millipore filter just before injection. The push-pull cannulae, injection needles and polyethylene tubing were stored in 0.0013% benzalkonium chloride solution and flushed repeatedly with the solution to be injected just before use. Syringes and other glassware used were rendered pyrogen-free by autoclaving heating methods in an oven. Before an experiment began, a YSI 401 thermistor probe (Yellow Springs Instrument Co., Yellow Springs, Ohio) was inserted into the colon to a depth of 9-12 cm and held in place by surgical tape wrapped gently around the base of the tail. The temperature of each cat was recorded on a multi-channel potentiometric recorder for a baseline period of at least 1 hour. Animals were unrestrained throughout the experiments.

Histology

At the conclusion of a series of experiments ventricular injection sites were verified by the injection of 0.2 ml bromothymol blue. The sites of push-pull perfusion were verified according to standard histological procedures. The cat was killed by an overdose of pentobarbitone sodium and 10% buffered, neutral formalin was perfused

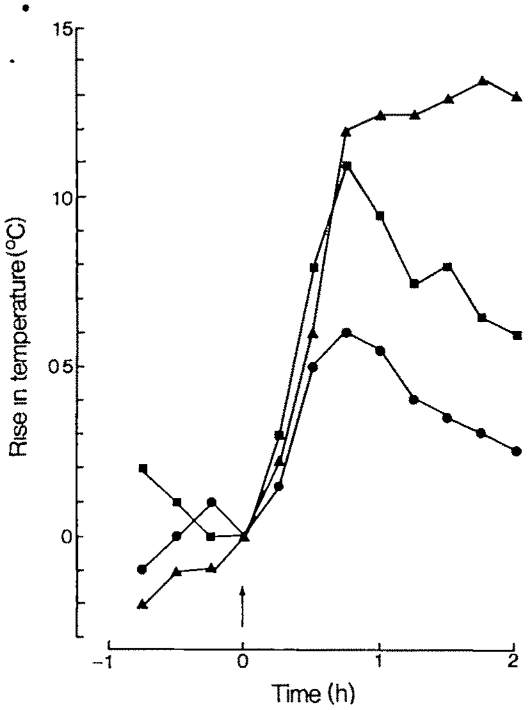

Figure 1 Hyperthermia induced in a cat by successive intraventricular injections (at arrow) of 1 (●), 4 (■) and 10 μg (▲) RX72601,

through the thoracic aorta after the heart was clamped. The brain was washed and blocked; the sections were then cut at 100 μm on a freezing microtome and stained for cells and fibres according to a procedure modified after Wolf (1971).

Results

Intraventricular injections of RX72601

When injected into the lateral ventricle in a volume of 0.2 ml, RX72601 produced a dose-dependent rise in the cat's temperature over a ten-fold dose range. Doses of less than 1 μg had very little effect on temperature. Doses greater than 20 μg were not investigated. Figure 1 shows the differences in the hyperthermia produced in a single cat when progressively larger doses were injected in successive experiments. Although the onset of the hyperthermic responses was usually between 1 and 10 min after the injection, the duration of the fever produced was often difficult to assess, especially with larger doses, because of the interference encountered from a non-specific fever

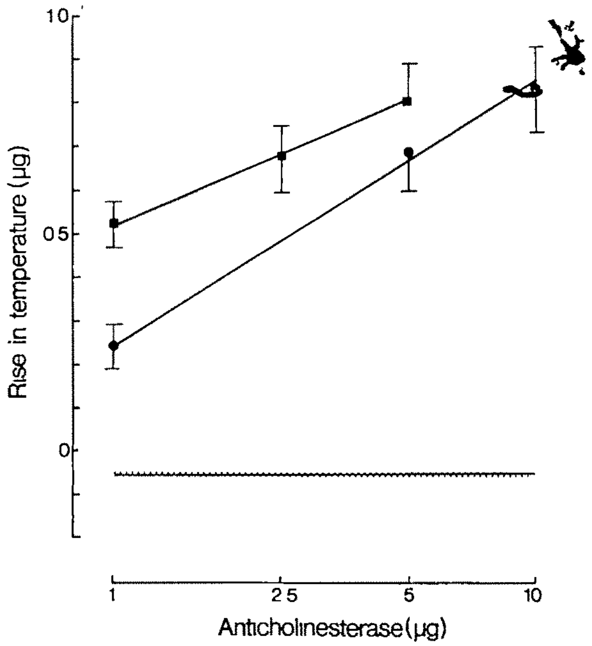

Figure 2 Hyperthermia induced in cats by the intraventricular injection of either RX72601 (●) or neostigmine (■). Each point is the mean from 4-6 experiments. Vertical bars show s.e. mean. Hyperthermia was measured as the maximum rise in temperature produced within 1 h of injection. The shaded area represents the effect (mean ± s.e.) produced by a similar injection of 5 ions.

of slow onset. This latter fever, which has been described by other workers (Feldberg, Myers & Veale, 1970; Feldberg & Saxena, 1970) and is thought to be due to an unknown pyrogenic factor, normally developed between 1 and 3 h after an injection. At such time the shorter duration hyperthermias produced by lower doses of RX72601 were completed, whilst the rises in temperatures produced by large doses of RX72601 were still in evidence. Figure 2 presents the relationship between the dose and maximum rise in temperature produced by RX72601 in the first hour after injection.

In a preliminary attempt to identify the anatomical region responsible for the drug-induced hyperthermia, 5 μg RX72601 was injected into the lateral ventricles in three different volumes. Figure 3 shows that the greatest hyperthermia was produced when this dose was injected in a dose volume of 0.2 ml. Subsequent injections of bromothymol blue demonstrated that 0.05 ml dye was contained within the lateral ventricle receiving the injection, that 0.1 ml dye was distributed

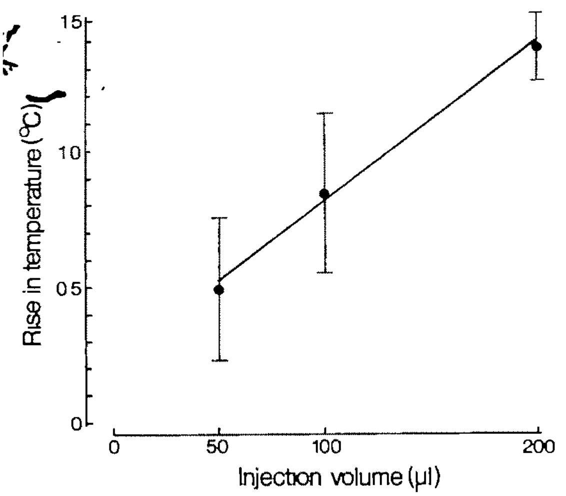

Figure 3 The effect of dose-volume on the hyperthermia produced by intraventricular injection of 5 μg RX72601. Each point represents the mean of 4 experiments. Vertical bars show s.e. mean.

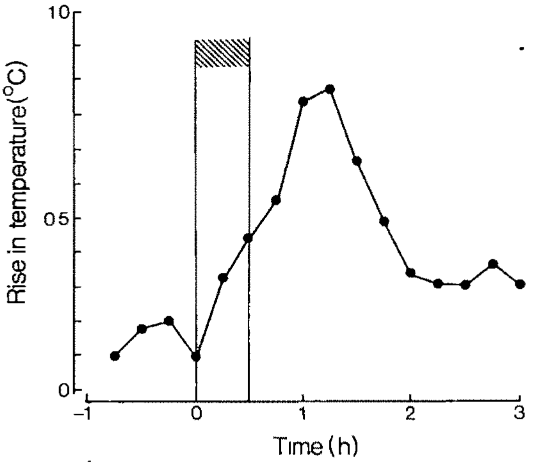

Figure 4 Change in body temperature of an unanaesthetized cat produced by a push-pull perfusion bilaterally of RX72601 (1.0 μg/min) for 30 minutes. The sites in the dorsal hypothalamus were AP 11.0 as indicated in Figure 5 by the triangles (▲)

throughout the lateral ventricle and part of the third ventricle, whilst 0.2 ml dye stained the whole of the lateral and third ventricles and sometimes extended caudally through the aqueduct into the fourth ventricle. Thus the main site of action for RX72601 appeared to be the structures which line the walls of the lateral and third ventricles.

Although the intensity of the symptoms observed varied on different occasions, the hyperthermia produced by the intraventricular injection was invariably accompanied by a behavioural syndrome. The symptoms produced included flattening of the ears and growling; no other responses of fear or aggression (Norton, 1969) were produced, however, and the animal could be handled without provoking an attack. Intense preening or scratching of the face and pinnae with the forepaws as if irritation was being experienced, was also observed. Higher doses (10-20 μg) ordinarily produced pronounced shivering and sometimes mydriasis and nystagmus. The onset of the behavioural effects coincided with the onset of hyperthermia and they persisted intermittently for about 60-75 minutes. In contrast to the effect on body temperature, there appeared to be no diminution in the magnitude of the behavioural effects when RX72601 was injected in a smaller dose or volume. As with the body temperature effects, RX72601 had little discernible effect on behaviour at doses below 1 μg.

Intraventricular injections of neostigmine

To ascertain that the responses observed after the intraventricular injection of RX72601 were not independent of its anticholinesterase properties, the effect of another anticholinesterase, neostigmine, which is structurally unrelated to RX72601, was also tested. Figure 2 shows that a dose-related hyperthermia was also produced by neostigmine although the slope of the dose-response line is different from that of RX72601. Neostigmine also produced a similar behavioural syndrome to RX72601. In addition, the neostigmine-treated animals consistently resisted handling, although there was no actual attack upon the experimenter.

Push-pull perfusion

In order to localize more precisely the site of action for RX72601 a series of push-pull perfusions were performed at discrete tissue sites within the diencephalon and telencephalon. Perfusion with RX72601 in a concentration of 0.125 μg/min at any site failed to produce either hyperthermia or behavioural changes. When the concentration of RX72601 in the perfusing fluid was increased to 0.5-1.0 μg/min, some sites were identified at which perfusion of this concentration for 30 min produced approximately 0.75° C rise in temperature. However, such hyperthermic responses appeared to be site specific for other sites

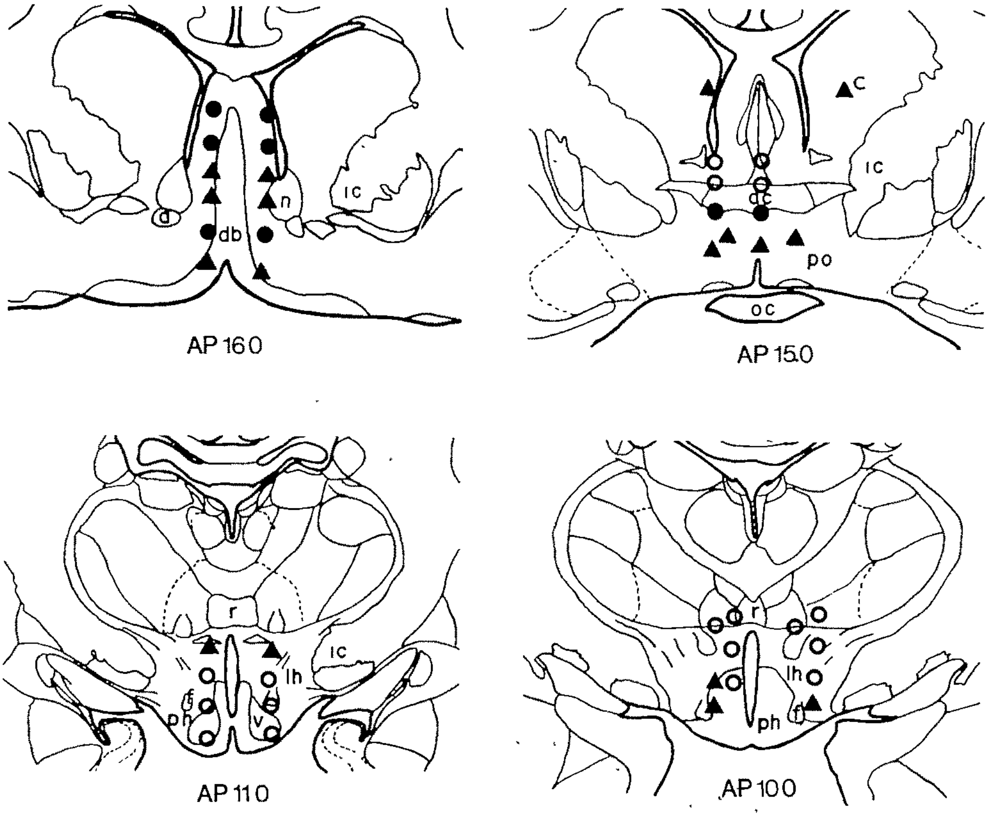

Figure 5 Representative frontal sections of cat brain illustrating the anatomical sites at which a push-pull perfusion of RX72601 produced a rise in temperature. (▲) Indicates sites where push-pull perfusion of 1.0 μg/min RX72601 produced hyperthermia of 0.4° C or greater; (○) Indicates sites where doses of drug 0.125-0.5 μg/min were ineffective; (●) indicates sites where 1.0 μg/min was ineffective. Key to labelled structures; a, nucleus accumbens; ac, anterior commissure; c, caudate nucleus; db, diagonal band of Broca; f, fornix, ic, Internal capsule; lh, lateral hypothalamus, n, nucleus of anterior commissure; oc, optic chiasm; ph, posterior hypothalamus; po, pre-optic area; r, nucleus reuniens; v, ventromedial hypothalamus.

perfused with this concentration showed only minimal (0.2-0.3° C) rises in body temperature. An example of a marked rise in temperature produced by perfusion of RX72601 at a site in the dorsal hypothalamus is illustrated in Figure 4.

Figure 5 indicates that the sites most sensitive to RX72601 were in the rostral hypothalamus, the ventral region of the septum, the caudate nucleus, the posterior region of the hypothalamus and at an area situated at the junction between the hypothalamus and the mid-brain at the level of AP10. However, in contrast to the intense behavioural syndrome observed after the intraventricular injection of RX72601, perfusion of the anticholinesterase at tissue sites failed to produce more than minimal behavioural effects.

Discussion

On the basis of previous experiments in which cholinomimetic agonists were injected directly into cerebral tissue, Myers (1971a, 1974) has proposed that a cholinergic system located in the hypothalamus and mid-brain is involved in thermogenesis in the cat. The results obtained from the present experiments, in which the concentration of endogenous ACh in CNS structures is raised by inhibiting the enzyme responsible for its metabolic degradation, support this proposal. Such cholinergic thermogenesis has also been reported for other species. Both Myers & Yaksh (1969) and Hall & Myers (1971) have reported that hyperthermia is produced by micro-injection of cholinomimetic agonists into the hypothalamus of the monkey. Avery (1970) has observed a similar cholinergically-induced rise in temperature in rats, although in this species hypothermia has also been reported by other workers after CNS administration of cholinomimetic agents (for review see Myers, 1974).

Results obtained from both types of experiments designed to locate the anatomical site or sites at which RX72601 acted to provoke hyperthermia support the concept that the cholinergic heat production pathway is diffuse in nature. Although sites in both the rostral and caudal hypothalamus appear to be critically involved in the hyperthermia, activity at the other sites lining the ventricles cannot be ruled out at this stage. Three further pieces of information are pertinent to this last point. Firstly, the compound is quaternary in nature and so will not cross biological membranes easily. This implies that such sites will be close to the ventricular walls. Secondly, when a cholinomimetic substance is injected directly into the caudate nucleus, hyperthermia has previously been reported (Connor, Rossi & Baker, 1966). Thirdly, in the present experiments, the magnitude of hyperthermia produced by a push-pull perfusion of RX72601 at diencephalic sites never equalled the intensity of the response produced by an intraventricular injection implying, perhaps, that there is more than one site of action in the latter case. A more extensive study of push-pull perfusions or micro-injections will be necessary to define all of the anatomical sites at which RX72601 exerts its hyperthermic action.

Besides the effect on thermoregulation, an injection of RX72601 into the cerebral ventricles produced changes in the emotional behaviour of the cat. The animals crouched down with flattened ears and growled almost continuously for the first 50-60 min after the injection. Thereafter, the symptoms became intermittent and gradually decreased both in frequency and intensity. Intense face wiping, scratching or preening often accompanied the behavioural symptoms. A higher dose of RX72601 produced mydriasis and nystagmus. This duration of action corresponds to the duration of acetylcholinesterase inhibition previously measured *in vivo* (Dettmar *et al.*, 1974b).

Much work has been done on cholinergically induced emotional behaviour since the original report (Myers, 1964) that micro-injections of carbachol or ACh into the cat's hypothalamus produced an affective defence reaction. The results obtained in the present study differ from some of the earlier results (for review see Myers, 1974) in two ways. Firstly, the spectrum of emotional behaviour observed during the present experiments is more limited than that previously reported. Secondly, such emotional behaviour as was observed occurred after intraventricular injection rather than push-pull perfusion at the selected diencephalic sites, as would perhaps have been expected from previous investigations.

Several explanations are possible for these differences. The doses of RX72601 used may have been too low to provoke the full behavioural syndrome, although a small number of subsequent experiments with higher doses do not confirm this suggestion. In addition, results from earlier work in both animals (Metcalf & Dettmar, 1975; G. Metcalf, G. Shilcock & M.H. Smith, unpublished observations) and man (Dettmar *et al.*, 1974b) when applied to the present experiments suggest that the doses used would cause virtually complete inhibition of acetylcholinesterase in the restricted areas of drug application. Alternatively, most of the previous studies have used carbachol as the cholinomimetic agent; this drug is known to stimulate both muscarinic and nicotinic receptors in the periphery, and Myers (1974) has suggested that behavioural responses produced by this drug may result from stimulation of CNS receptors with muscarinic characteristics. By comparison, the results obtained previously with RX72601 suggest that this compound may be more active at nicotinic sites (Dettmar *et al.*, 1974a, b). Thus the latter drug may exhibit a different spectrum of activity. The more complete aggressive syndrome observed after injection with neostigmine lends at least some credence to this latter explanation. Finally, it may be that the manipulation of the endogenous level of ACh offers an approach closer to physiological reality than the injection of synthetic cholinomimetic agonists resistant to normal metabolic degradation. Further experiments will be necessary to evaluate these possibilites.

This research was supported in part by grant GB 35380 from the National Science Foundation and by U.S. Office of Naval Research contract N-00014-67-A-0226-0003. Dr M.J. Readhead of the Pharmaceutical Division, Reckitt & Colman, Ltd, Hull, England, kindly supplied the RX72601. G.M. was visiting Assistant Professor in Psychobiology from Hull, England, and is grateful to the Wellcome Trustees for their award of a travel grant. P.C.R. was a David Ross pre-doctoral fellow of the Purdue Research Foundation. Reprint requests to R.D.M.

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(Received December 18, 1974.
Revised April 1, 1975.)

Br. J. Pharmac. (1975), 55, 17-25

EFFECTS OF IPRINDOLE ON RESPONSES OF SINGLE CORTICAL AND CAUDATE NEURONES TO MONOAMINES AND ACETYLCHOLINE

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1 The technique of microelectrophoresis was used to study the effects of iprindole on single neurones in the cerebral cortex and caudate nucleus of the rat.

2 Iprindole, when applied for a brief period, did not affect the firing rate of the vast majority of neurones tested.

3 Both potentiation and antagonism of neuronal responses to noradrenaline, dopamine, and 5-hydroxytryptamine could be observed after a brief application of iprindole. Potentiation and antagonism often occurred after the same application of iprindole, antagonism always preceding potentiation.

4 Responses to acetylcholine were affected by iprindole similarly: both potentiation and antagonism of the responses could be observed.

5 Responses to glutamate were not affected by iprindole.

6 It is concluded that the potentiation of responses to monoamines by iprindole cannot be explained on the basis of uptake blockade; this potentiation may be due to the blockade of masked receptors on the post-synaptic cell.

7 It is suggested that the common pharmacological action of the tricyclic antidepressants may be the ability to block both monoamine and acetylcholine receptors in the brain.

Introduction

According to the monoamine theory of affective disorders, the tricyclic antidepressant drugs exert their antidepressant effect by potentiating the pharmacological actions of monoamines at postsynaptic receptor sites in the brain (Schildkraut, 1965; Davis, 1970). It is generally believed that the monoamine potentiating effect of the tricyclic antidepressants is due to their ability to block the uptake of monoamines into monoamine-containing nerve terminals (Iversen, 1974). Previous reports on imipramine and desipramine seem to confirm this hypothesis. It has been shown that these antidepressants block the uptake of noradrenaline (NA) and of 5-hydroxytryptamine (5-HT) into brain tissue (Ross & Renyi, 1967; 1969), and that they also potentiate neuronal responses to NA and 5-HT (Bradshaw, Roberts & Szabadi, 1974). However, there are reports suggesting that the 'uptake blockade hypothesis' of potentiation may not apply to another antidepressant, iprindole.

Iprindole is a tricyclic antidepressant drug with a similar structure and clinical antidepressant efficacy to imipramine (Imlah, Murphy & Mellor, 1968, Rickels, Chung, Csanalosi, Sablosky & Simon, 1973). Like imipramine, iprindole potentiates the peripheral effects of NA (Gluckman & Baum, 1969). However, in contrast to imipramine and other tricyclic antidepressant compounds, iprindole does not block the uptake of NA into sympathetically innervated tissues (Gluckman & Baum, 1969; Lahti & Maickel, 1971). It has also been reported that, in contrast to imipramine and desipramine, iprindole is ineffective in blocking the uptake of NA (Ross, Renyi & Ögren, 1971; Rosloff & Davis, 1974), dopamine and 5-HT (Ross *et al.*, 1971) into brain tissue. However, it is not known how pharmacological responses to the monoamines are affected by iprindole in the brain.

We have reported earlier that the tricyclic antidepressants imipramine and desipramine have a dose-dependent dual effect on responses of single cortical neurones to NA, 5-HT and acetylcholine (ACh): smaller doses potentiate, and bigger doses antagonize the responses (Bradshaw *et al.*, 1974; Bevan, Bradshaw & Szabadi, 1975a). In the experiments described here we used the technique

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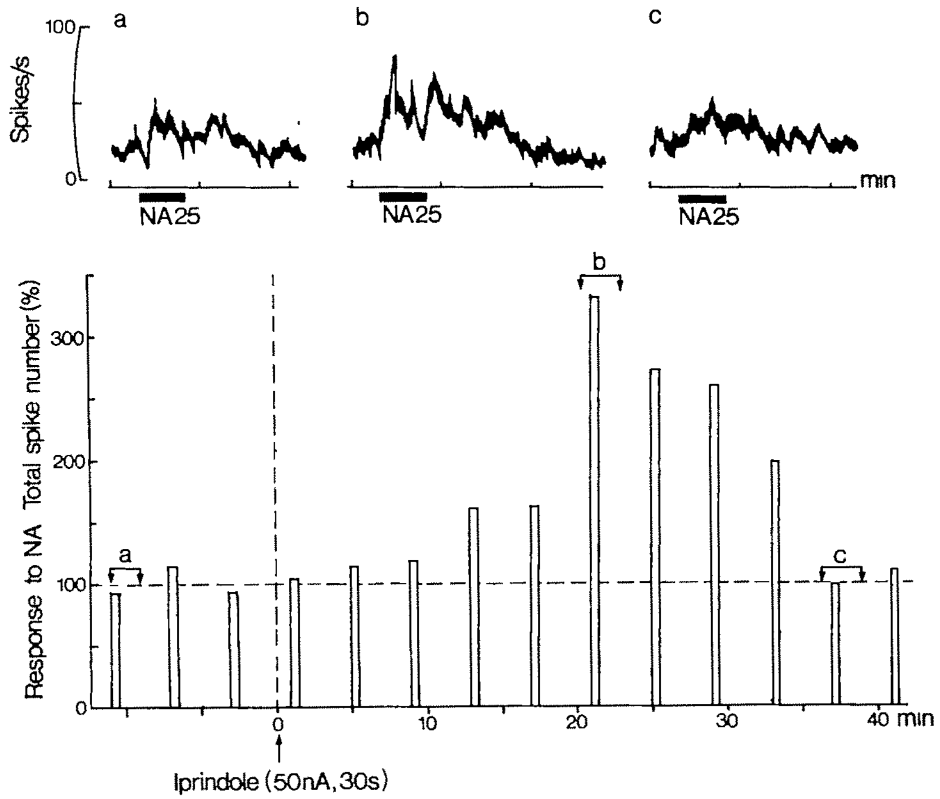

Figure 1 Potentiation of excitatory responses of a single cortical neurone to noradrenaline (NA) by iprindole. Top of the figure shows excerpts from the ratemeter recording of the firing rate of the neurone. Horizontal bars indicate applications of NA; numbers refer to the intensity of the ejecting current (nA). (a) Control response to NA. (b) Potentiated response to NA 21 min after a brief application of iprindole (50 nA; 30 seconds). (c) Recovery of control response 37 min after the application of iprindole. The graph below shows the time course of the entire study. The sizes of the responses to NA are expressed as a % of the mean of the control responses. Each column represents a single response. Letters above the graph indicate responses illustrated in the ratemeter tracings above.

of microelectrophoresis in order to investigate how responses of single cortical and caudate neurones to NA, dopamine, 5-HT and ACh can be modified by iprindole.

Some of these results have been communicated to the British Pharmacological Society (Bevan, Bradshaw & Szabadi, 1975b).

Methods

Male albino Wistar rats, weighing between 250 and 300 g were used. Anaesthesia was induced with halothane (3.0%) and maintained with halothane (0.5-1.0%) delivered from a temperature and flow-rate compensated vapourizer (Fluotec Mk 3, Cyprane Ltd.). All animals respired spontaneously via a tracheal cannula. ECG and EEG were monitored continuously throughout the experiment. Rectal temperature was maintained between 37° C and 38° C with a heating pad controlled by a thermosensitive rectal probe.

The head of the animal was held rigidly in a stereotaxic frame. A small hole was prepared in the skull (coordinates: A: 8 mm; L: 2.4 mm; König & Klippel, 1963), and a small area of the cortex exposed according to the method of Bradshaw & Szabadi (1972). A six-barrelled micropipette was then introduced into the brain, under microscopic control. Neurones were studied both in the cerebral cortex and in the underlying caudate nucleus, using the same electrode track.

Six-barrelled glass micropipettes were constructed and filled as described by Bradshaw, Roberts & Szabadi (1973). Two barrels of each micropipette contained 4 M NaCl, one barrel for recording action potentials, the other for use in current balancing. The remaining barrels contained drug solutions. The following drug solutions were used: noradrenaline bitartrate (0.1 M, pH 3 0-3.5), dopamine hydrochloride (0.1 M, pH 4.0-4.5), 5-hydroxytryptamine bimaleate (0.1 M, pH 3.5), acetylcholine chloride (0.1 M, pH 3.6), sodium glutamate (0.05 M, pH adjusted to 8.5 by the addition of 0.1 M NaOH), and iprindole hydrochloride (0.1 M, pH 4.6).

Standard techniques were used for recording action potentials, and for the electrophoretic application of drugs. A cumulative record of the total number of action potentials was obtained as described by Bradshaw, Szabadi & Roberts (1973).

All the neurones studied were spontaneously active. All the drugs were applied by microelectrophoresis. Repeated responses to an agonist were compared before and after a brief application of iprindole. In order to ensure that standard ejecting current pulses gave rise to standard pulses of drug ejection (Bradshaw *et al.*, 1973), the intervals between drug applications were kept constant by the use of a sequential timing device (Bevan & Bradshaw, 1973). A retaining current of 25 nA was passed between drug ejections. Cells were excluded from drug interaction studies if the variation in the size of control responses to an agonist exceeded ± 10% (see Bradshaw *et al.*, 1974).

The magnitude of a response was measured by calculating the difference between the number of spikes generated during the response and the number of spikes generated during an equivalent period when no drug was applied ('total spike number'; see Bradshaw *et al.*, 1974).

Results

Cerebral cortex

Direct effect on neuronal firing The effect of iprindole on the firing rate was studied in 68 cells. The dose of iprindole applied was 30-100 nA passed for 30-60 seconds. Four cells responded with a clear increase in firing rate to the application of iprindole, whereas 18 cells were depressed. There was no significant correlation between the dose of iprindole applied and the effect on neuronal firing. The excitation or depression was always of a temporary nature, and the original base-line firing rate recovered within a minute after the application of iprindole had been terminated. On 46 cells iprindole did not affect the firing rate. On occasions a reduction in spike amplitude was observed; such cells were not used for drug interaction studies.

Effect on responses to noradrenaline

Excitatory responses Both potentiation and antagonism of excitatory responses to NA could be observed after a brief application (30-100 nA for 30-60 s) of iprindole

Potentiation was seen in 6 cells. A response was regarded as potentiated if there was more than 20% increase over the size of the mean control response (Bradshaw *et al.*, 1974). An example of potentiation is shown in Figure 1. On one cell, the response was first reduced in size following the application of iprindole; this antagonism was followed later by potentiation, and finally by recovery of the control response. (A response was regarded as antagonized if there was more than 20% decrease in the size of the response compared to the mean of the control responses). On 2 cells no significant change in the size of responses could be observed after the application of iprindole. The degrees of potentiation and antagonism seen in each cell are summarized in Figure 6.

Depressant responses Both potentiation and antagonism of depressant responses to NA could be observed after a brief application of iprindole. Potentiation was seen in 10 cells, antagonism was seen in 3 cells. In 2 cells both antagonism and potentiation could be observed; in both cases antagonism preceded potentiation. An example of the potentiation of the depressant response to NA by iprindole is shown in Figure 2. In 2 cells no significant change in the size of responses could be observed after the application of iprindole. The results are summarized in Figure 6.

Effect on responses to dopamine

Excitatory responses Both potentiation and antagonism of excitatory responses to dopamine could be observed after a brief application of iprindole. Potentiation was seen in 5 cells, antagonism was seen in 4 cells. In 3 cells both antagonism and potentiation of the response could be observed; antagonism always preceded potentiation. Figure 3 shows an example of antagonism followed by potentiation. In one cell no significant change in the size of responses could be observed after the application of iprindole. The results are summarized in Figure 6.

Depressant responses Potentiation was seen in 3 cells depressed by dopamine; antagonism was observed in one cell. In one cell no effect of iprindole could be observed. Figure 6 summarizes the results.

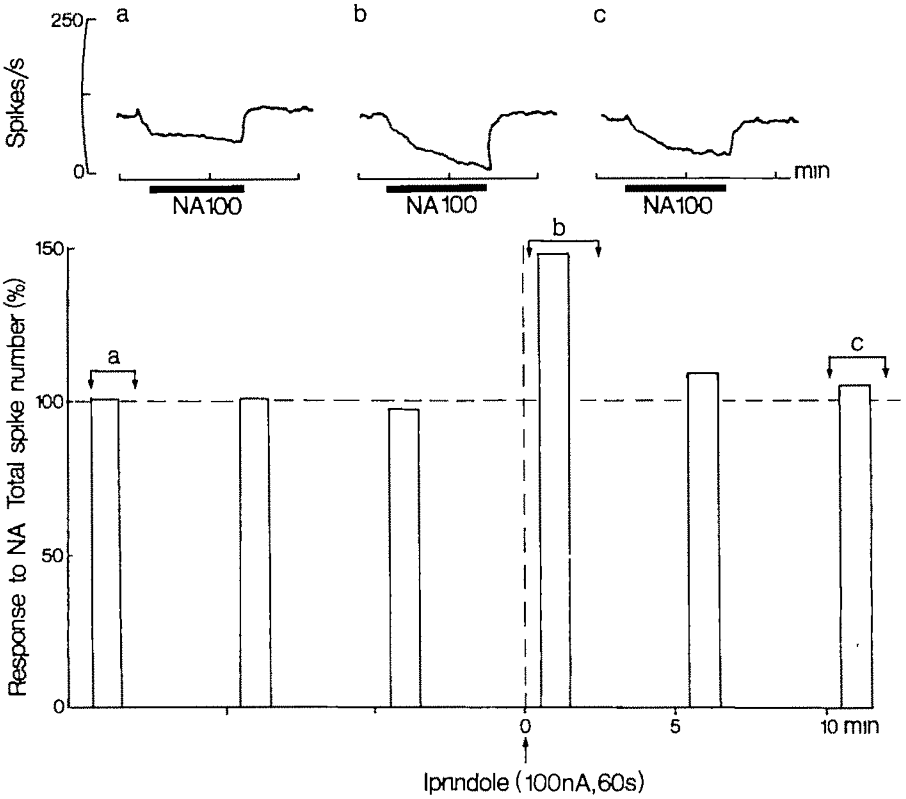

Figure 2 Potentiation of depressant responses of a single cortical neurone to noradrenaline (NA) by iprindole. Top of the figure shows excerpts from the ratemeter recording of the firing rate of the neurone (as in Figure 1). (a) Control response to NA. (b) Potentiated response 1 min after a brief application of iprindole (100 nA; 60 seconds). (c) Recovery of the control response 11 min after the application of iprindole. Graph below shows the time course of the entire study (as in Figure 1).

Effect on responses to acetylcholine Both potentiation and antagonism of excitatory responses to ACh could be observed after a brief application of iprindole. Potentiation was observed in 3 cells, antagonism was seen in 5 cells. In 3 cells both antagonism and potentiation could be seen; the antagonism always preceded the potentiation. An example of antagonism followed by potentiation is shown in Figure 4. In one cell, iprindole had no significant effect. The data are summarized in Figure 6.

Effect on responses to glutamate The effect of iprindole on excitatory responses to glutamate was studied in 10 cells. The effects of glutamate on the firing rate were studied for 20-30 min following the application of iprindole. In none of the cells could any significant change be observed in the size of the response to glutamate after the application of iprindole (see Figure 6).

Caudate nucleus

The direct effect of a brief application of iprindole was tested in 18 neurones: 3 cells were excited, 2 cells were depressed, whereas in 13 cells there was no change in the firing rate. The effect of iprindole was also examined on neuronal responses to NA, dopamine and 5-HT. The same patterns of drug interaction could be observed as described above for the cerebral cortex. Following a brief application of iprindole, both antagonism and potentiation of excitatory responses to NA (2 cells), dopamine (3 cells) and 5-HT (3 cells) could be observed. Similarly, both antagonism and potentiation of depressant responses to NA (3 cells) and dopamine (5 cells) were seen. Excitatory responses to glutamate (3 cells) were not affected by iprindole. Figure 5 shows an example of the potentiation of depressant responses to dopamine by iprindole in the caudate nucleus.

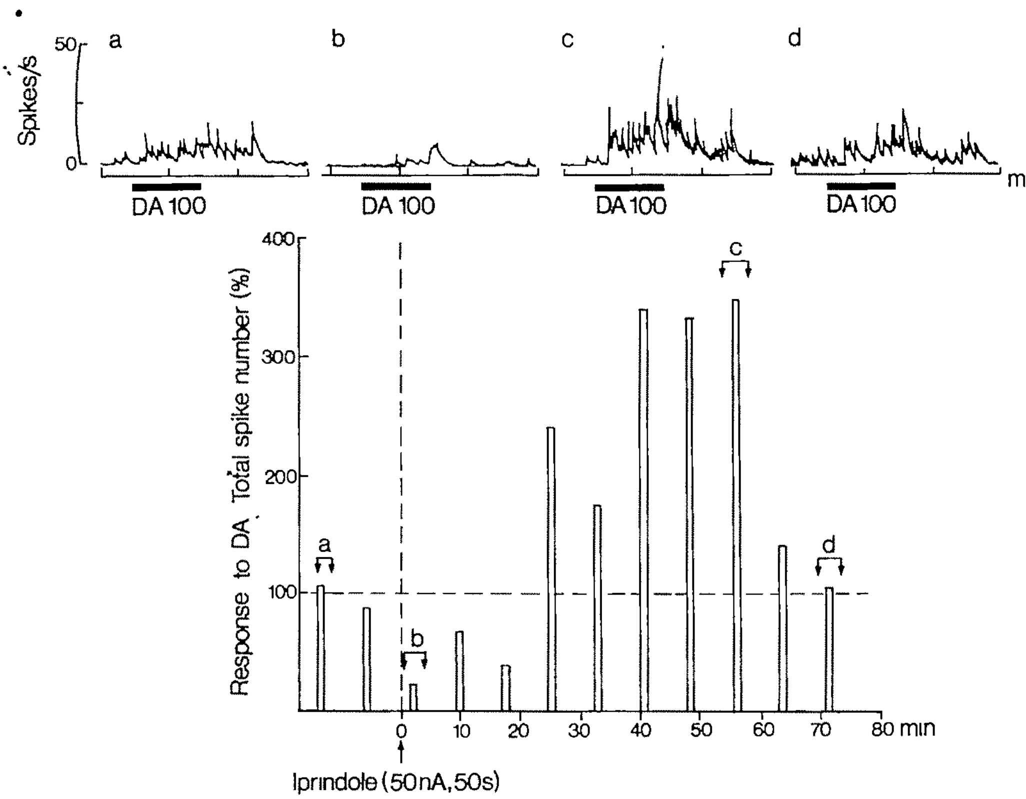

Figure 3 Antagonism and potentiation of excitatory responses of a single cortical neurone to dopamine (DA) by iprindole. Top of the figure shows excerpts from the ratemeter recording of the firing rate of the neurone (as in Figure 1). (a) Control response to DA. (b) Antagonized response, 1 min after a brief application of iprindole (50 nA; 50 seconds). (c) Potentiated response, 55 min after the application of iprindole. (d) Recovery of the control response 71 min after the application of iprindole. Graph below shows the time course of the entire study (as in Figure 1).

Discussion

A brief application of iprindole did not affect the firing rate of the majority of neurones tested in the cerebral cortex and in the caudate nucleus. However, in a small number of cells a response to iprindole was observed. A possible explanation for this effect of iprindole itself could be that it reflects the interaction between endogenously released monoamine transmitters and iprindole. As responses to ACh are also affected by iprindole, an interaction with ACh released by cholinergic inputs to the neurone should also be considered. Like iprindole, imipramine and desipramine can also evoke both excitatory and depressant responses in spontaneously firing cortical neurones (Bradshaw *et al.*, 1974). In a few cells, a reduction in spike amplitude was observed in response to iprindole. This probably reflects the local anaesthetic action of tricyclic antidepressant drugs (Domenjoz & Theobald, 1959).

We have found that iprindole can cause both antagonism and potentiation of the responses of cortical and caudate neurones to monoamines (NA and dopamine in the cerebral cortex; NA, dopamine and 5-HT in the caudate nucleus). When both antagonism and potentiation of the response occurred after a single application of iprindole, antagonism invariably preceded the development of potentiation (see Figure 3). After a brief ejecting pulse, the concentration of iprindole probably rises quickly to a peak, and then gradually declines (Bradshaw *et al.*, 1974) so that antagonism of the response to the monoamine is likely to reflect a higher, and potentiation a lower concentration of the antidepressant (see Bradshaw *et al.*, 1974).

The most plausible explanation for antagonism

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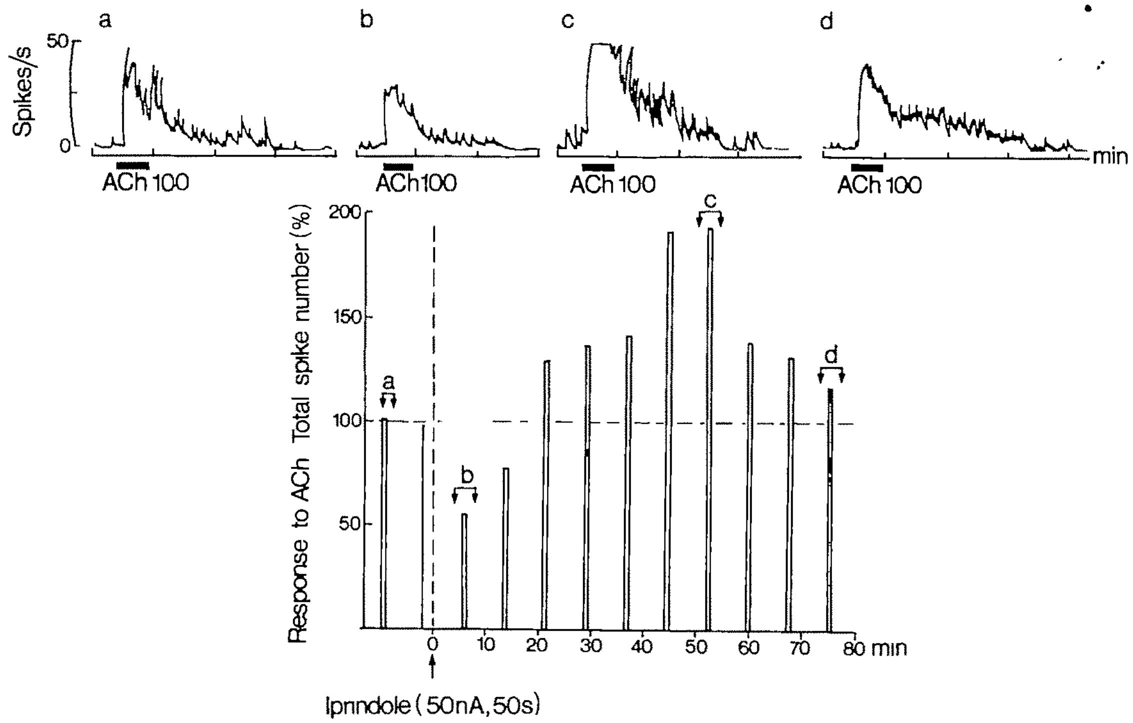

Figure 4 Antagonism and potentiation of excitatory responses of a single cortical neurone to acetylcholine (ACh) by iprindole. Top of the figure shows excerpts from the ratemeter recording of the firing rate of the neurone (as in Figure 1). (a) Control responses to ACh. (b) Antagonized response, 6 min after a brief application of iprindole (50 nA; 50 seconds). (c) Potentiated response, 52 min after the application of iprindole. (d) Recovery of the control response 75 min after the application of iprindole. Graph below shows the time course of the entire study (as in Figure 1).

is the blockade of monoamine receptors on the post-synaptic neurone. This would indicate that iprindole probably shares the α-adrenoceptor blocking and anti-5-HT actions of other tricyclic antidepressants (Domenjoz & Theobald, 1959, Callingham, 1966).

It is more difficult to interpret the potentiation of neuronal responses to monoamine by iprindole. As iprindole is ineffective in blocking the uptake of NA, dopamine and 5-HT into brain tissue (Ross *et al.,* 1971), uptake blockade cannot be an explanation for the potentiation observed in our experiments. It is possible that not only antagonism, but also potentiation is due to a post-synaptic effect of iprindole. We have proposed earlier that the dose-dependent dual effect of peripheral monoamine antagonists (e.g. methysergide, sotalol) (Bevan, Bradshaw & Szabadi, 1974; Bevan, Bradshaw, Roberts & Szabadi, 1974), and of tricyclic antidepressants (e.g. imipramine, desipramine) (Bradshaw *et al.,* 1974) may be due to the selective blockade of one or both of two opposing receptor populations on the post-synaptic neurone (Szabadi & Bradshaw, 1974). According to this hypothesis, a smaller concentration of the antagonist (or antidepressant) would block selectively the more sensitive masked (opposite) receptors on the cell, thus causing potentiation of the response, whereas a higher concentration of the antagonist (or antidepressant) would block the dominant receptors as well, thus causing antagonism of the response (Szabadi & Bradshaw, 1974; Bradshaw *et al.,* 1974). The dual effect of iprindole on neuronal responses to monoamines may also be interpreted by this model.

We have found that iprindole has a dual effect not only on responses to monoamines, but also on responses to ACh: both antagonism and potentiation can be observed. As the appearance of antagonism invariably precedes the development of potentiation, it is likely that antagonism reflects a higher, and potentiation a lower concentration of iprindole (see above, and Bevan

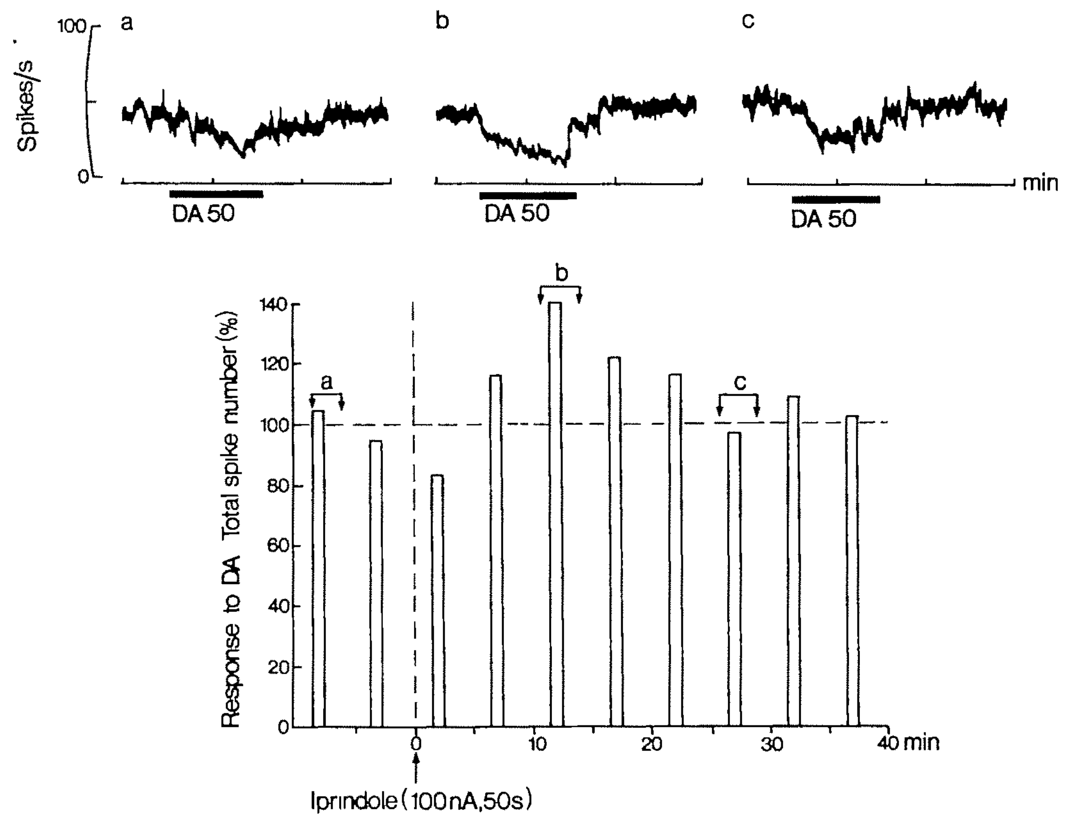

Figure 5 Potentiation of depressant responses of a single caudate neurone to dopamine (DA) by iprindole. Top of the figure shows excerpts from the ratemeter recording of the firing rate of the neurone (as in Figure 1). (a) Control response to DA. (b) Potentiated response to DA 12 min after a brief application of iprindole (100 nA; 50 seconds). (c) Recovery of the control response 27 min after the application of iprindole. Graph below shows the time course of the entire study (as is Figure 1).

et al., 1975a). Imipramine, desipramine and atropine have a similar dual effect on responses to ACh (Bevan *et al.,* 1975a). As in the case of imipramine, desipramine and atropine, the antagonism can be interpreted as a blockade of muscarinic receptors. It has been reported that iprindole blocks the effects of ACh in peripheral test systems, although it is less potent than atropine or imipramine (Gluckman & Baum, 1969). We have proposed earlier that potentiation of neuronal responses to ACh by imipramine, desipramine and atropine may be due to the selective blockade of masked inhibitory receptors (Bevan *et al.,* 1975a). This model could account for the potentiating effect of iprindole on responses to ACh.

Although inprindole differs from imipramine and desipramine in that it does not block the uptake of monoamines into brain tissue (Ross *et al.,* 1971), it shares the ability with imipramine and desipramine of potentiating and antagonizing neuronal responses to monoamines and ACh (Table 1). In contrast to the antidepressants, the monoamine antagonists methysergide and sotalol potentiate and antagonize responses to monoamines without affecting responses to ACh, whereas the cholinergic antagonist atropine potentiates and antagonizes responses to ACh without affecting responses to monoamines (see Table 2 in Bevan *et al.,* 1975a). As both antagonism and potentiation may be interpreted in terms of post-synaptic receptor blockade (see above), a common feature of the antidepressants may be the ability to block both monoamine and

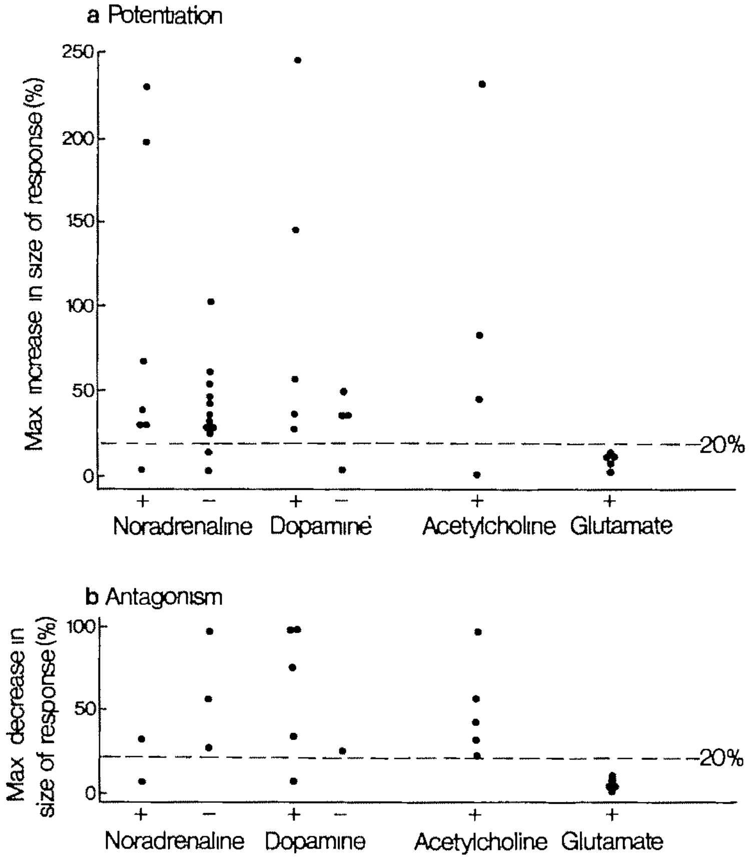

Figure 6 Summary of the effects of iprindole on responses of single cortical neurones to dopamine, noradrenaline, acetylcholine and glutamate. (a) Potentiation. each point shows the maximum potentiation observed in one individual cell (e.g. response (b) in Figure 1). + = excitatory responses; – = depressant responses A response was regarded as potentiated if there was more than 20% increase over the size of the control response (see text). (b) Antagonism each point shows the maximum degree of antagonism observed in one individual cell (e.g. response (b) in Figure 3). A response was regarded as antagonized if there was more than 20% decrease over the size of the control response (see text).

Table 1 Interaction between tricyclic antidepressants and potential neurotransmitters on single neurones in the mammalian brain

	Noradrenaline	*Dopamine*	*5-Hydroxytryptamine*	*Acetylcholine*	*Glutamate*
Cat—cerebral cortex					
Imipramine	P, A (1)	—	P, A (1)	P, A (2)	—
Desipramine	P, A (1)	—	P, A (1)	P, A (2)	0 (1)
Rat—cerebral cortex					
Iprindole	P, A	P, A	—	P, A	0
Rat—caudate nucleus					
Desipramine	P, A (3)	P, A (3)	P, A (3)	P, A (3)	0 (3)
Iprindole	P, A	P, A	P, A	—	0

P = potentiation; A = antagonism; 0 = not affected; — = not studied.
(1) Bradshaw, Roberts & Szabadi (1974); (2) Bevan, Bradshaw & Szabadi (1975a); (3) Bevan, Bradshaw & Szabadi (1975c).

ACh receptors in the brain. However, it remains to be determined whether other tricyclic compounds with a similar structure but with no marked antidepressant efficacy (e.g. phenothiazines) share this ability.

This work was supported by the Scottish Home and Health Department and the Mental Health Trust and Research Fund. P.B. gratefully acknowledges the receipt of a training award from the M.R.C. Iprindole was the generous gift of John Wyeth & Brother Ltd. We are grateful to Mr R. Lamb for his technical assistance.

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(Received March 18, 1975.
Revised May 12, 1975.)

Br. J. Pharmac. (1975), 55, 27-40

THE ACTION OF 5-HYDROXYTRYPTAMINE ON CHEMORECEPTOR DISCHARGES OF THE CAT'S CAROTID BODY

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1 Chemoreceptor discharges were recorded *in vivo* from fine filaments of the carotid sinus nerve containing a single or several active units; their frequency was used as an index of receptor activity. The effects of 5-hydroxytryptamine (5-HT) on chemoreceptors were studied in 26 adult cats. At times, sinus baroreceptor discharges were recorded from the carotid nerve and the effect of 5-HT on the discharges was examined.

2 Intra-carotid injections of 5-HT (2-20 μg) induced a sharp and brief increase in chemoreceptor discharges, followed by depression or block which lasted for several seconds. Repeated injections at short intervals, and a small dose after a large dose of 5-HT resulted in depressed or blocked response to 5-HT.

3 5-HT in high doses (10-20 μg, i.a.) slightly depressed the chemoreceptor discharges induced by either acetylcholine (ACh) or NaCN, when these substances were applied within 20 s after 5-HT. 5-HT (5-20 μg, i.a.) applied during asphyxia induced a further increase in chemoreceptor discharges, soon followed by block of the discharges lasting for several seconds.

4 Atropine or hexamethonium in high doses did not change the chemoreceptor response to 5-HT, while that to ACh was markedly depressed.

5 (+)-Lysergic diethylamide (LSD), methysergide or gramine did not alter the response to 5-HT, while LSD in low doses produced a marked increase in chemoreceptor discharges..

6 Acute and chronic treatment with reserpine (5-10 mg/kg, i.v.) of the animals did not change the sensitivity and the reactivity of the chemoreceptor to ACh and NaCN, while the chemoreceptor response to 5-HT was augmented, indicating an increase in the sensitivity of chemoreceptors to 5-HT.

7 5-HT in small doses (2-10 μg, i.a.) induced a marked increase in sinus baroreceptor discharges; subsequently discharges were depressed or blocked for several seconds.

8 The results are discussed in relation to possible mechanism of action of 5-HT on the chemoreceptors. It is concluded that the exogenous 5-HT probably acts directly on the chemosensory nerve endings and depolarizes them, but 5-HT contained in the carotid body does not play a significant role in the generation of chemoreceptor discharges.

Introduction

Catecholamines and indolamine are known to be normally present in the carotid body of different mammalian species (Lever, Lewis & Boyd, 1959; Niemi & Ojala, 1964; Fillenz & Woods, 1966; Hamberger, Ritzen & Wersäll, 1966; Chiocchio, Biscardi & Tramezzani, 1966, 1967; I-Li Chen, Yates & Duncan, 1967; Zapata, Hess, Bliss & Eyzaguirre, 1969; Kobayashi, 1971; Chiocchio, King, Carballo & Angelakos, 1971). Whether they play a role in initiating or modulating chemoreceptor activity of the carotid body is still not clear. Some authors have indicated that catecholamines may not have a significant role in chemoreceptor function (Eyzaguirre & Koyano, 1965; Eyzaguirre & Zapata, 1968; Zapata *et al.*, 1969), although other evidence indicates otherwise (Biscoe, 1965; Sampson, 1972; Andrew, Black, Comroe & Jacobs, 1972). A physiological role for 5-hydroxytryptamine (5-HT) may not be discarded, since the carotid body contains a certain amount of 5-HT (Chiocchio *et al.*, 1967, 1971); furthermore, histochemical methods have shown that 5-HT is located in the electron-dense core vesicles of the Type I cells (Chiocchio *et al.*, 1967; I-Li Chen *et al.*, 1967). These findings lend some support to the view that 5-HT may have some functional role in the carotid body. However, reports on the effects of 5-HT on carotid body chemoreceptors have been contradictory. Some authors have reported pronounced chemoreceptor stimulation by 5-HT (Douglas & Toh, 1952; Ginzel & Kottegoda, 1954, McCubbin, Green, Salmoiraghi & Page, 1956; Comroe & Mortimer, 1964), while others have failed to observe such an effect (cf Heymans & Neil, 1958, Eyzaguirre & Koyano, 1965). More recently, Andrew *et al.* (1972) have shown a species difference in the response of chemoreceptors to 5-HT; the agent stimulates carotid body chemoreceptors and produces reflex hyperpnoea in dogs, but not in cats.

In most experiments done by previous investigators, reflex changes in respiration were used as indicators of chemoreceptor activity of the carotid body. However, changes in respiration after 5-HT do not seem to be the most sensitive indicator of chemoreceptor activity, since these effects may be complicated by other neurogenic or circulatory influences (cf. Comroe, 1966, Black & Torrance, 1971; Jacobs & Comroe, 1971).

The present experiments were designed to examine the direct action of 5-HT on carotid body chemoreceptor discharges in an attempt to clarify the discrepancies previously reported.

Methods

The methods employed in the present experiments were essentially similar to those described in a previous paper (Nishi & Eyzaguirre, 1971).

Twenty-six adult cats were anaesthetized with sodium pentobarbitone (40 mg/kg, i.p.). The trachea was cannulated for artificial ventilation. A positive pressure respirator (Harvard Apparatus, 660) was set to provide an arterial $P\text{O}_2$ of approximately 90 mmHg, and a $P\text{CO}_2$ of 40 mmHg, and both the $P\text{O}_2$ and $P\text{CO}_2$ were periodically measured during the experiment, using Astrup micro-equipment (Radiometer D616). To maintain constant ventilation throughout an experiment, pneumothorax was made by opening a hole in the thoracic cavity. Both vago-sympathetic trunks and the left carotid sinus nerve were sectioned to prevent respiratory reflex movements arising from either injection of chemicals or asphyxia (Heymans & Neil, 1958). The right carotid nerve was exposed and cut at its junction with the glossopharyngeal nerve. The nerve was cleaned of surrounding connective tissue, and prepared for recording. Baroreceptor activity was eliminated by cutting or crushing the nerve branches going to the sinus region. In a few cases, a filament containing baroreceptor activity was selected to examine the effects of injected chemical substances on impulse conduction. Sympathetic nerves going to the carotid sinus region were severed to avoid possible efferent effects.

The peripheral end of the carotid nerve was dissected into fine filaments containing either a single or a few active units. Nerve impulses were led off from the nerve with platinum bipolar electrodes, amplified for display on an oscilloscope (Tektronix 5103N), and simultaneously led to an FM-Data recorder (Sony PFM-5). The nerve impulses were led to an impulse height discriminator, whose output pulses were fed to a counter-D-A converter system (Yokokawa, Hewlett & Packard 5302A, 5311A). Nerve impulses were counted for 0.2 or 1 s periods at 100 μs intervals. Arterial blood pressure was recorded from the femoral artery by an electronic manometer (Nihonkoden MT-4). Its output and that of the D-A converter were displayed on an ink-writing recorder, while impulses were monitored on a cathode-ray oscilloscope. The nerve impulses and pressure records were registered on different channels of the data-recorder for subsequent analysis.

A small polyethylene catheter was inserted into the right common carotid artery through a muscular branch of the artery, the tip of the catheter being placed about 2 mm caudal to the origin of the superior thyroid artery. Chemical substances were injected into the common carotid artery through the catheter, or into the femoral vein. In the case of an intra-arterial injection, all chemicals were dissolved in Locke solution (pH 7.3) at 34°-35° C; the volume of the injected solution was 0.25 ml. The doses used are expressed as the salt. The substances employed were: acetylcholine chloride (Daiichi Seiyaku), sodium cyanide (Merck), 5-hydroxytryptamine creatinine sulphate complex (Wako Pure Chemicals), creatinine sulphate (Wako Pure Chemicals), (−)-methyl-(+)-lysergic acid butanolamide (methysergide) (Sandoz), (+)-lysergic acid diethylamide (LSD) (Sandoz), gramine hydrochloride (Wako Pure Chemicals), atropine sulphate (Merck) and reserpine (Boehringer-Mannheim). All experiments were conducted at room temperatures of 20°-25° C.

Results

Chemoreceptor discharges were identified by. (1) making intra-arterial injections of a small amount of NaCN (2 μg), which increased the discharge

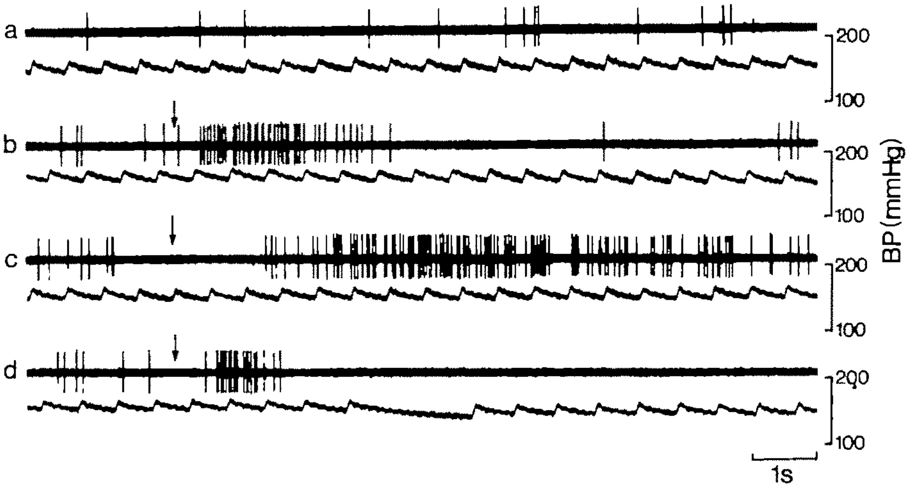

Figure 1 Effect of intra-arterial injections of acetylcholine (ACh), NaCN and 5-hydroxytryptamine (5-HT) on single chemoreceptor fibre discharge in the cat. Upper traces show impulse activity and lower ones, arterial blood pressure. The animal was artificially ventilated with room air and the resting rate of the discharge of the unit was 2-4 s at an arterial Po_2 of 94 mmHg and a Pco_2 of 41 mmHg (a). ACh (2 μg in 0.25 ml Locke solution) (b), and NaCN (2 μg in 0.25 ml Locke solution) (c) elicited a considerable increase in chemoreceptor discharges. In (d) 5-HT (2 μg in 0.25 ml Locke solution) was given. Injections were made at arrows.

frequency 2-3 s after the injections (Nishi & Eyzaguirre, 1971), (2) ventilating the animal with pure O_2, which caused a decrease or abolition of spontaneously appearing discharges.

In animals ventilated with room air, the resting discharge frequency of single units ranged from 0-12 impulses/s at an arterial Po_2 of 85-98 mmHg. ACh or NaCN, given intra-arterially, evoked threshold responses in doses of 0.5-2 μg; the response to ACh was transient and disappeared within 5 s, while NaCN produced a gradual increase in chemoreceptor discharges

The action of 5-hydroxytryptamine on the chemoreceptor discharge

In all cases 5-HT was injected intra-arterially and caused a fall in blood pressure. This effect appeared after a latent period of 5-7 s from the start of the injection and lasted for 10-20 seconds. The fall in blood pressure following the intra-arterial injection of 5-HT was depressed or abolished, after the nodose ganglion was excised. The results are consistent with those obtained by Andrew *et al.* (1972). The depressor response is most probably due to excitatory effects of 5-HT on the nodose ganglion, which in turn, produces depressor reflexes (Jacobs & Comroe, 1971). The records of Figure 1 illustrate a typical unitary response to ACh, NaCN and 5-HT. Each substance elicited a marked increase in chemoreceptor discharges. When 5-HT (2 μg) was delivered through the artery, the discharges abruptly increased with a latency of about 0.5 s (measured from the start of the injection to the onset of discharges) (Figure 1d). This excitatory effect of 5-HT was transient, and disappeared within 2 s; it was followed by depression of the spontaneously occurring discharges. Thereafter, the discharge frequency gradually returned to the control level. In most instances, the response of chemoreceptor discharges to intra-arterial injections of 5-HT consisted of two phases; an initial transient increase in discharge frequency followed by a decrease or complete block of the spontaneously occurring discharges. Occasionally, during the course of an experiment, some units showed a change in their response pattern to 5-HT; after a decrease in the discharge frequency, the discharges again started to increase gradually, returning to the control level within 10 seconds.

When a given dose of 5-HT was injected a number of times within a short period, the response to the second dose varied depending on the intervals at which the injections were delivered. In most cases, the response to the second dose was greatly reduced, and after an initial larger dose, a previously effective smaller dose was without effect for a longer period (Figure 2). The first injection of 5-HT (2 μg, i.a.) induced a sharp transient increase in discharges, while the second one delivered 6 s later did not elicit a

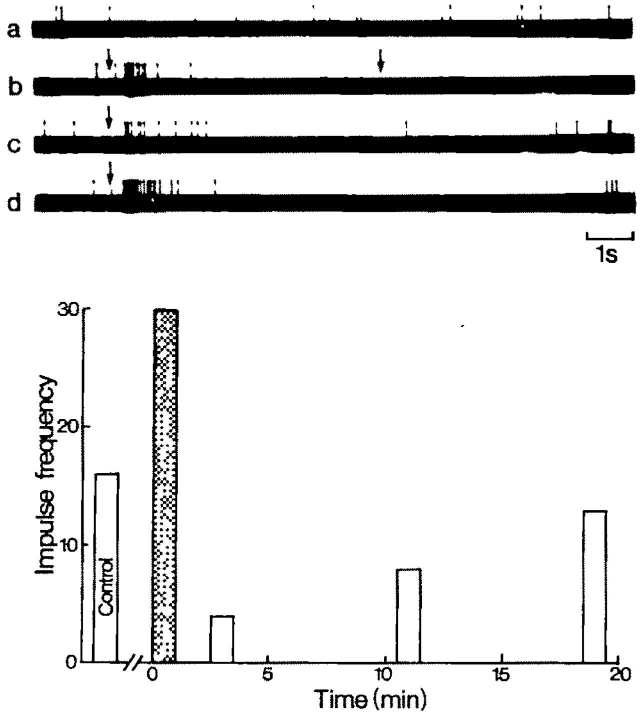

Figure 2 Effect of repeated intra-arterial injections of 5-hydroxytryptamine (5-HT) on chemoreceptor activity; (a)-(d) show effects of repeated injections of 5-HT on single chemoreceptor fibre discharges. (a) Spontaneous discharge during control period; (b) 5-HT (2 μg in 0.25 ml Locke solution) applied successively at arrows, (c) 5-HT (2 μg) injected 5 min after the second injection of 5-HT in (b); (d) 5-HT (2 μg) 15 min after 5-HT in (c). Injections were made at arrows.

Lower graph illustrates effects of a large dose of 5-HT on subsequent chemoreceptor responses to a small dose of 5-HT. Open bars represent the number of chemoreceptor discharges in a few active units-preparation induced by 5-HT (2 μg in 0.25 ml Locke solution), and the dotted bar that induced by 5-HT (20 μg in 0.25 ml Locke solution). 5-HT (20 μg) was applied 30 min after the control 5-HT.

response (Figure 2b). The third injection, 5 min after the second one, produced a slight increase in the discharge (Figure 2c), while spacing the 5-HT deliveries at longer intervals (15 min in Figure 2d) produced a response similar to that obtained during the control period. The effect of a large dose of 5-HT on the responses to small doses subsequently applied, are illustrated in the lower graph of Figure 2, where the discharge frequency from a few units preparation was counted during 5 s after the start of the injections of 5-HT. The depressant effect of the large dose of 5-HT on the responses to smaller doses lasted for about 30 minutes.

The relationship of the two components of the biphasic response to 5-HT varied with different doses of the agent. On increasing the doses to 20 μg, the number of chemoreceptor discharges occurring within a few seconds after the injections, increased, but the dose-response curve appears S-shaped, while the period during which spontaneous discharges were blocked or depressed was prolonged proportionally to the dose of 5-HT. A quantitative estimate of effects of increasing doses of 5-HT on chemoreceptor discharges is shown in Figure 3, where the upper records were taken from discharges of a single chemoreceptor unit responding to different doses of 5-HT; in the lower graph dose-response curves were constructed from discharges of a few active unit preparations.

Removal of superior cervical ganglion 5-HT in small doses (0.5-5 μg) injected into the common carotid artery stimulates the superior cervical ganglion in the cat (Trendelenburg, *1958*, *1959*; Jaramillo & Volle, 1968). Stimulation of the sympathetic ganglia by 5-HT could release catecholamines from the sympathetic nerve endings in the carotid body, which in turn, could cause a decrease in blood flow through the carotid

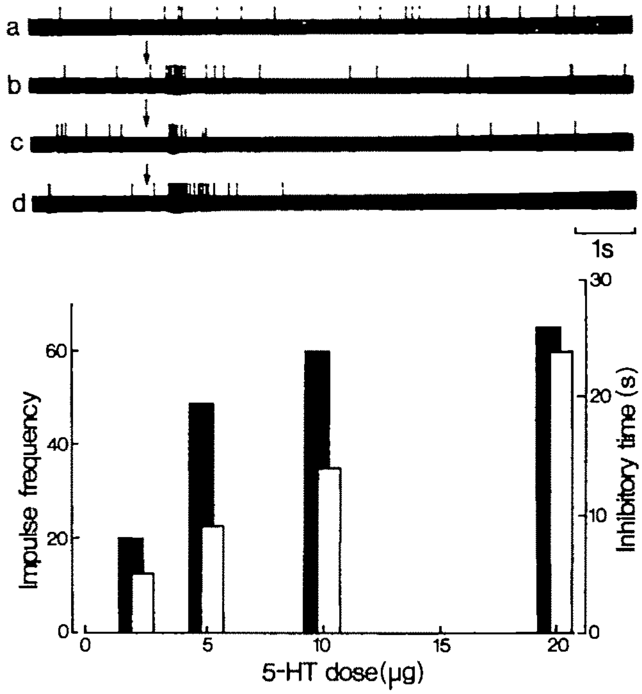

Figure 3 Chemoreceptor responses evoked by different doses of 5-hydroxytryptamine (5-HT); (a)-(d) show effects of intra-arterial injections of different doses of 5-HT on single chemoreceptor fibre discharges. (a) Spontaneous discharge in control period; (b) 5-HT (2 μg in 0.25 ml Locke solution); (c) 10 μg; (d) 20 μg. Injections were made at arrows every 20 minutes. Lower graph illustrates dose-response curves to 5-HT. Since there was tachyphylaxis to 5-HT, different doses of 5-HT were given every 20 minutes. The total number of chemoreceptor discharges of a few active units was counted during 5 s after the injections, and is shown by black columns, while the time during which the discharge frequency decreased below the control level after 5-HT is indicated by open columns.

body. This could lead to increased chemoreceptor activity. Furthermore, dopamine, which may be released from the sympathetic nerve endings (Thoenon, Haefely & Gey, 1967) could act directly on chemoreceptors and cause a decrease in chemoreceptor discharges, since this agent has been reported to decrease or block chemoreceptor discharges from the carotid body in the cat (Sampson, 1972). If this was the case, the present results could have been due primarily to a stimulating action of 5-HT on the superior cervical ganglion.

To clarify this possibility the effects of 5-HT on chemoreceptor discharges were examined after the right superior cervical ganglion had been removed. 5-HT injected intra-arterially induced a change in chemoreceptor discharges, which was identical to that obtained in the animals with intact superior cervical ganglia. Thus, the action of 5-HT on chemoreceptor discharges is not due to secondary effects mediated through sympathetic stimulation.

Effects of 5-hydroxytryptamine antagonists Since 5-HT may affect the local carotid body circulation by either a constrictor or dilator effect which could affect chemoreceptor activity, methysergide, LSD and gramine hydrochloride which are very potent antagonists of the effects of 5-HT on peripheral and muscular receptors of the agent (see Gyermek, 1966), were employed to prevent possible effects of 5-HT on the vessels of the carotid body.

Effects of LSD and methysergide on the responses of chemoreceptor discharges to 5-HT are shown in Figure 4. The upper records (a)-(c) were taken from a several-units preparation and the lower ones were obtained from a different animal. An intra-arterial injection of 5-HT produced a transient increase in discharge frequency and later abolished the spontaneous discharges (Figure 4a). LSD (10 μg, i.a.) delivered 20 min after the previous injection of 5-HT, elicited a marked increase in the discharges (Figure 4b). An

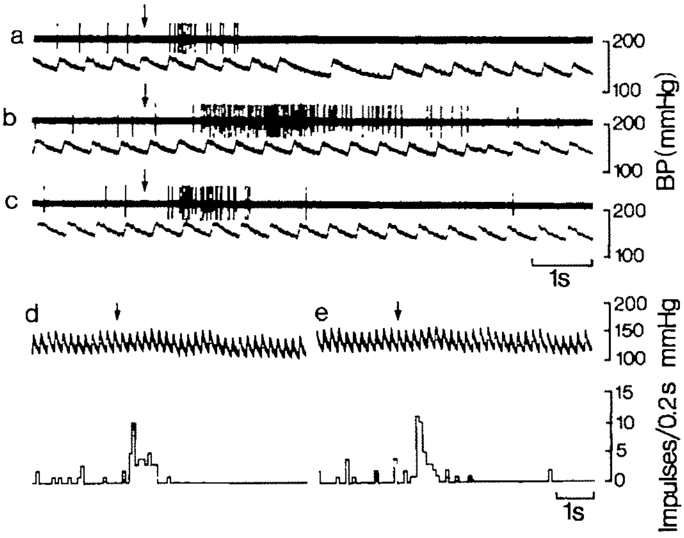

Figure 4 Effect of (+)-lysergic acid diethylamide (LSD) and (−)-methyl-(+)-lysergic acid butanolamide (UML) on chemoreceptor response to 5-hydroxytryptamine (5-HT). (a)-(c) Show effects of 5-HT on single chemosensory fibre discharges before and after LSD. Injections made at arrows. (a) 5-HT (5 μg in 0.25 ml Locke solution, i.a.); (b) LSD (10 μg in 0.25 ml Locke solution, i.a.); (c) 5-HT (5 μg in 0.25 ml Locke solution, i.a.) 5 min after LSD (100 μg/kg, i.v.). Tracings in (a)-(c) as in Figure 1. See text. (d)-(e) Show chemoreceptor responses to 5-HT before and after UML. Upper tracings show arterial blood pressure changes and lower ones, discharge frequency in a few active chemoreceptor units. Injections made at arrows. (d) 5-HT (5 μg in 0.25 ml Locke solution, i.a.) applied 30 min before UML; (e) 5-HT (5 μg in 0.25 ml Locke solution, i.a.) applied 5 min after UML (100 μg/kg, i.v.).

intravenous injection of LSD (100 μg/kg) after the previous injection in (b) also caused a gradual increase in the discharge frequency (not illustrated). Shortly after the intravenous injection of the agent, 5-HT was delivered. The response of chemoreceptor discharges to 5-HT was practically identical to that shown in the control, while the change in blood pressure caused by 5-HT was prevented by LSD (Figure 4c). LSD in doses up to 100 μg/kg did not affect the excitatory and inhibitory components of the response of chemoreceptors to 5-HT.

Intra-arterial or intravenous injections of methysergide (10-100 μg, i.a., 50-100 μg/kg, i.v.), and gramine (50 μg, i.a., 50 μg/kg, i.v.) did not produce any change in the frequency of spontaneous chemoreceptor discharges. The responses of chemoreceptor discharges to 5-HT before and after methysergide (100 μg/kg, i.v.) are shown in Figure 4d and e. Neither methysergide nor gramine altered the response to 5-HT.

The experiments just described are not conclusive with regard to possible vascular effects of 5-HT in the carotid body. However, they indicate that the effects of 5-HT on chemoreceptor discharges are probably due to a direct action on mechanisms responsible for the initiation of chemoreceptor discharges.

Effects of atropine and hexamethonium It has been proposed that ACh plays an important role in the generation of chemoreceptor discharges in the carotid body (Eyzaguirre & Zapata, 1968; Eyzaguirre, Nishi & Fidone, 1972). If this is also the case with regard to 5-HT, there is a possibility that the action of 5-HT on chemoreceptors may be mediated through a cholinergic mechanism. Atropine and hexamethonium were used to examine effects of cholinergic blocking agents on 5-HT responses.

Since large doses of atropine or hexamethonium can block or depress the responses to ACh and NaCN, when these agents are applied shortly after the cholinergic blocking agents (Nishi & Eyzaguirre, 1971), the same doses of cholinergic blocking agents were employed in the present experiments. Shortly after intravenous injections of either atropine (2-5 mg/kg) or hexamethonium (10-30 mg/kg), a small dose of 5-HT was injected intra-arterially (Figure 5). Intra-arterial injections of ACh and 5-HT produced a transient increase in the discharges during the control period (Figure

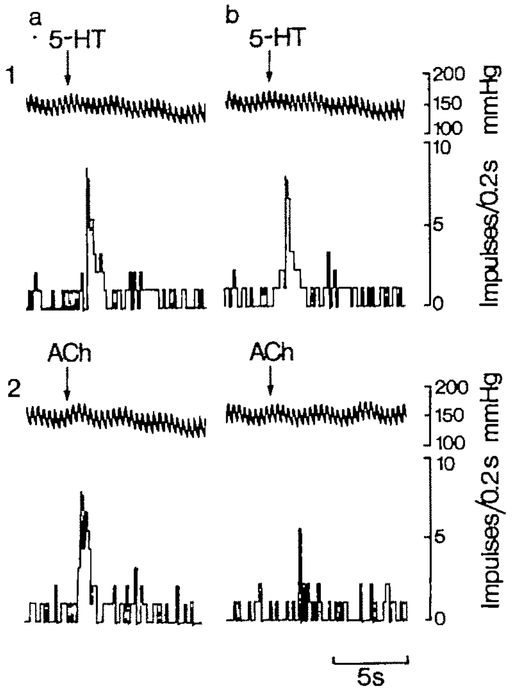

Figure 5 Effect of atropine on chemoreceptor response to 5-hydroxytryptamine (5-HT) and acetylcholine (ACh); (a) before; (b) 5-7 min after atropine (5 mg/kg, i.v.). Upper traces show arterial blood pressure and lower traces chemoreceptor discharge frequency in a few active units. Injections made at arrows. (a1) and (b1), 5-HT (2 μg in 0.25 ml Locke solution, i.a.); (a2) and (b2), ACh (5 μg in 0.25 ml Locke solution, i.a.).

5a). Five minutes after atropine (5 mg/kg, i.v.), the same doses of ACh and 5-HT used in the control were given. The response to 5-HT remained unchanged, while that to ACh was greatly depressed (Figure 5b).

The results are consistent with earlier observations *in vivo* where in the presence of atropine or hexamethonium the reflex response of respiration to 5-HT remained unaltered, while neither nicotine nor ACh caused any change in respiration (Ginzel & Kottegoda, 1954). Thus, it may be concluded that a cholinergic mechanism is not involved in eliciting the response of chemoreceptor discharges to 5-HT.

Creatinine sulphate Krnjević (1965) has noted that creatinine has a depressant action on some cortical neurones, while in other regions, e.g. the brain stem, the agent is mainly an excitant (Bradley & Wolstencroft, 1965). Thus it was possible that the creatinine moiety contained in

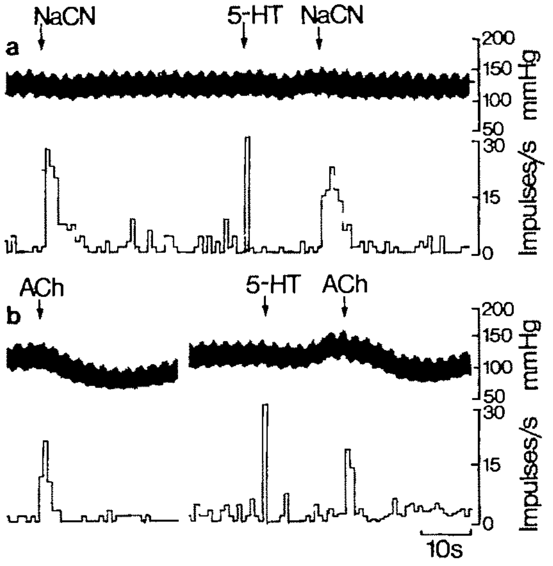

Figure 6 Effect of 5-hydroxytryptamine (5-HT) on chemoreceptor response to acetylcholine (ACh) and NaCN. Upper traces show arterial blood pressure changes. Lower traces, discharge frequency in a few active chemoreceptor units. (a) NaCN (2 μg in 0.25 ml Locke solution, i.a.) and 5-HT (20 μg in 0.25 ml Locke solution, i.a.) injected at arrows; (b) ACh (2 μg in 0.25 ml Locke solution, i.a.) and 5-HT (20 μg in 0.25 ml Locke solution, i.a.) injected at arrows.

5-HT creatinine sulphate complex employed in the present experiments could have participated in producing the responses to 5-HT. To study this possibility, the effects of creatinine sulphate on chemoreceptor discharges were examined in four different animals.

In all cases creatinine sulphate (0.5-20 μg, i.a.) did not produce any change in chemoreceptor discharge frequency, while a slight fall in blood pressure was observed after injections of relatively large doses of the agent, indicating that the response to 5-HT obtained in the present experiments can be attributed only to the 5-HT moiety.

Effects of 5-hydroxytryptamine on the responses to acetylcholine, NaCN and asphyxia

The effects of 5-HT on the responses of chemoreceptor discharges to ACh and NaCN were examined in five different cats. ACh and NaCN injected intra-arterially produced a marked increase in chemoreceptor discharges. After the discharge frequency returned to the control level, 5-HT (20 μg, i.a.) was injected. Shortly after the injection of 5-HT, ACh and NaCN were injected

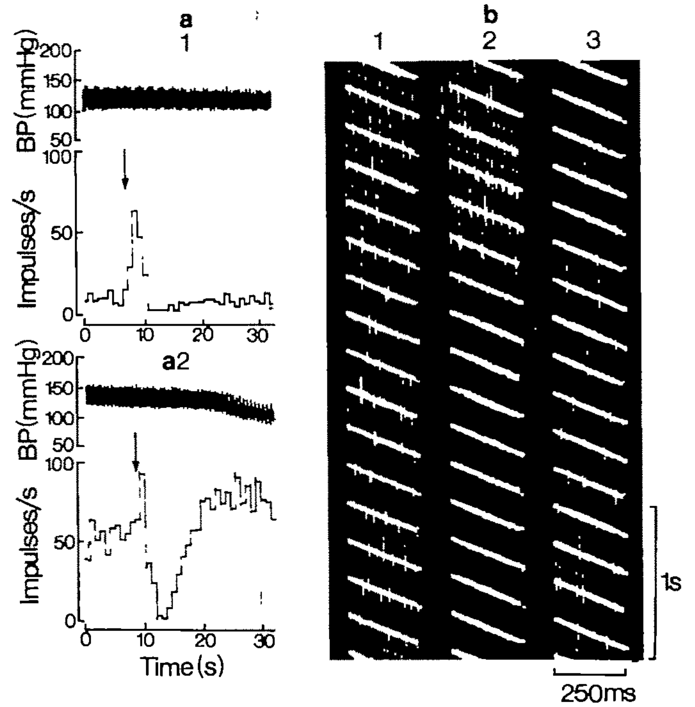

Figure 7 Effect of 5-hydroxytryptamine (5-HT) on chemoreceptor discharge during asphyxia (a1), 5-HT (2 μg in 0.25 ml Locke solution, i.a.) in control period, and (a2), same dose of 5-HT during asphyxia. Upper traces show arterial blood pressure changes. Lower traces, discharge frequency in a few active chemoreceptor units. Injections made at arrows. (b) Effect of 5-HT (5 μg in 0.25 ml Locke solution, i.a.) on single chemosensory fibre discharges during asphyxia: (b1, 2 and 3) continuous records. Injection made at arrow. Records read from top to bottom and left to right.

repeatedly every 5 minutes. The response to ACh or NaCN was slightly depressed when these substances were applied within 20 s after the injection of 5-HT. Otherwise, the response to ACh remained unchanged and subsequent injections of NaCN produced responses similar to control ones. A slight depression of the responses to ACh and NaCN observed immediately after 5-HT was relieved by larger doses of the agents. An example is illustrated in Figure 6. In Figure 6a, NaCN (2 μg) was injected intra-arterially 15 s after 5-HT (20 μg, i.a.), the response to NaCN being slightly depressed. Thirty minutes after the injection of 5-HT in Figure 6a, the same dose of 5-HT evoked a response similar to the previous one and ACh (2 μg), injected 17 s after the 5-HT, produced a response practically identical to that obtained during the control period (Figure 6b).

Figure 7 shows the effects of 5-HT on chemoreceptor discharges during asphyxia produced by stopping the artificial respirator in two different animals. During the control period, 5-HT (2 μg, i.a.) induced a marked increase in discharges followed by depression which lasted for about 8 s (Figure 7a(1)). One minute after the onset of asphyxia, when the discharges attained their maximal frequency, 5-HT (2 μg, i.a.) produced a further increase in discharges for a short period and thereafter, the discharge was depressed for about 10 s (Figure 7a(2)). Actual records obtained from a single-unit preparation in another cat also show both excitatory and inhibitory effects of 5-HT (5 μg, i.a.) on chemoreceptor discharges during asphyxia (Figure 7b(1), (2) and (3)). The inhibitory action of 5-HT on the discharges lasted for 10 s, the discharge frequency returning to the level before the 5-HT injection.

Thus, 5-HT had a transient inhibitory action on chemoreceptor responses to ACh, NaCN and asphyxia.

Effects of reserpine on responses to 5-hydroxytryptamine, acetylcholine and NaCN

Zapata *et al.* (1969) have shown that acute and chronic reserpine-treatment of cats does not change the sensitivity and reactivity of the carotid body to ACh, anoxic or asphyxic stimulation. Their results could suggest that 5-HT is not acting as a main 'excitatory transmitter substance' in the carotid body, since reserpine depletes 5-HT in various tissues (see Carlsson, 1966) and may have reduced the 5-HT content in the carotid body. However, 5-HT contained in the carotid body, may play a more subtle role in modulating a mechanism responsible for the initiation of chemoreceptor discharges. If endogenous 5-HT is acting on this mechanism, reserpine-treatment would be expected to increase the sensitivity to exogenous 5-HT in the carotid body, as reserpine sensitizes other preparations to catecholamines (Bein, 1956; Burn & Rand, 1958; Axelrod, 1964). Therefore, it was interesting to see whether reserpine changed the responses to 5-HT, ACh and NaCN.

In five cats, two or three nerve filaments containing a few active chemoreceptor units were selected and prepared for recording. In all cases, the right superior cervical ganglion was removed to prevent possible vascular effects on the circulation within the carotid body. After testing the sensitivity of chemoreceptor units to 5-HT, ACh and NaCN, one nerve filament was retained on the electrodes for continuous recording. Thereafter, reserpine (5-10 mg/kg) was injected intravenously and the responses of chemoreceptor units to 5-HT, ACh and NaCN were periodically examined for 20 hours. Some chemoreceptor units stopped discharging during the course of an experiment, probably because of deterioration of the nerve fibre in the filament. In such cases, the nerve filaments were discarded. Because of difficulty in obtaining a stable record for a long period, only five chemoreceptor units in two nerve filaments from two different cats survived for 20 hours.

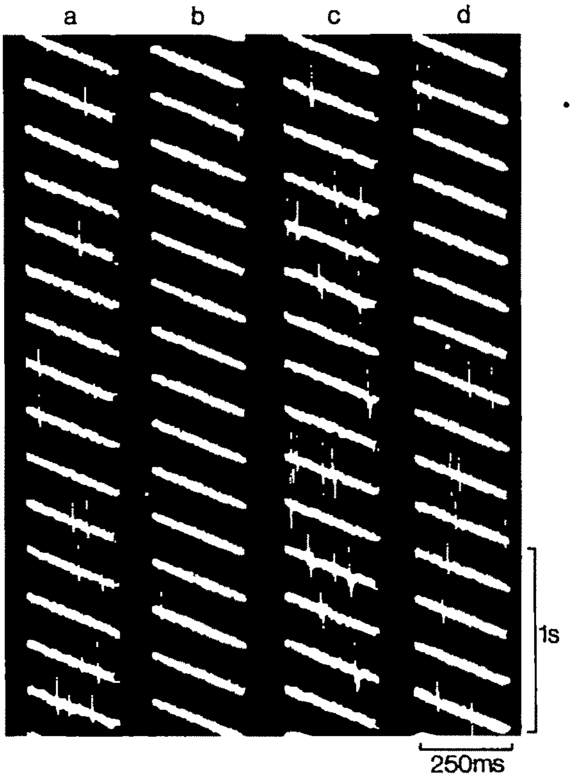

Figure 8 Effect of reserpine on chemoreceptor activity. Records (a)-(d) taken from a same filament containing two active chemoreceptor units. (a) Spontaneous discharge in control period; (b) 10 min after reserpine (10 mg/kg, i.v.); (c) 4 h after reserpine; (d) 20 h after reserpine.

Figure 8 illustrates an experiment in which chemoreceptor discharges of two units were recorded before and after reserpine. During the control period, the resting rate of discharges of each unit was 0-8 impulses/s and 2-5 impulses/s at an arterial $P\text{O}_2$ of 92 mmHg and a mean arterial blood pressure of 130 mmHg (Figure 8a). Immediately after an intravenous injection of reserpine (10 mg/kg), the spontaneous discharges disappeared, concomitant with a marked fall in blood pressure, and responses to ACh, NaCN and 5-HT were markedly depressed. The spontaneous discharges reappeared within 10 min, but the discharge frequency was low (Figure 8b). The depression of chemoreceptor discharges lasted for another 60 minutes. Thereafter, the discharge frequency gradually increased. Four hours after reserpine, the resting rate of the discharges was 5-12 impulses/s and 6-9 impulses/s at a $P\text{O}_2$ of 88 mmHg and a mean blood pressure of 98 mmHg (Figure 8c). ACh, NaCN and 5-HT induced pronounced responses of chemoreceptor discharges. Twenty hours after reserpine, blood pressure was still low (mean arterial pressure; 105 mmHg), but the resting rate of the discharges slightly decreased, compared with that observed 4-5 h after reserpine ($P\text{O}_2$; 87 mmHg) (Figure 8d). ACh and NaCN increased the chemoreceptor discharges, the response patterns to both chemicals being essentially similar to those obtained during the control period, while 5-HT inhibited the spontaneously appearing discharge after exerting a short excitatory effect.

Figure 9 illustrates another experiment in which chemoreceptor responses to 5-HT, NaCN and ACh were tested before and after reserpine. Control responses of three chemoreceptor units to 5-HT (2 μg, i.a.), NaCN (2 μg, i.a.) and ACh (5 μg, i.a.) are shown in column (a). Four hours after reserpine (5 mg/kg, i.v.), the responses to same doses of ACh and 5-HT used in the control period

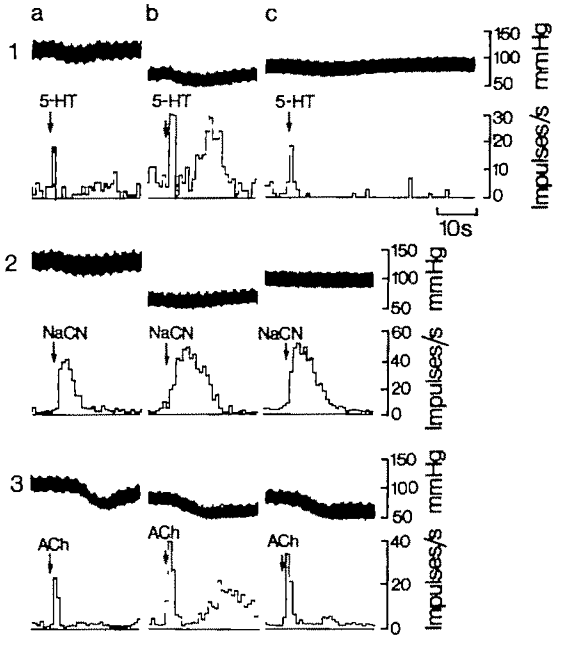

Figure 9 Chemoreceptor response to 5-hydroxytryptamine (5-HT), NaCN and acetylcholine (ACh) before and after reserpine. Upper traces show arterial blood pressure changes. Lower traces, discharge-frequency in a few active chemoreceptor units. Injections made at arrows. (a) Control; (b) 4 h after reserpine (5 mg/kg, i.v.); (c) 20 h after reserpine. (1), 5-HT (2 µg in 0.25 ml Locke solution, i.a.); (2) NaCN (2 µg in 0.25 ml Locke solution, i.a.); (3) ACh (5 µg in 0.25 ml Locke solution, i.a.). Records obtained from the same filament containing three chemoreceptor units before and after reserpine.

were very pronounced and NaCN also produced a long-lasting chemoreceptor stimulation (Figure 9b); this effect was probably influenced by changes in local circulation of the carotid body; because of the marked hypotension and bradycardia, the chemical substances may have reached and left slowly the sites responsible for initiating chemoreceptor discharges. Twenty hours after reserpine, both NaCN and ACh produced responses similar to those in the control, while 5-HT depressed the discharges for about 40 s, after inducing a sharp and transient increase in the discharges (Figure 9c). It is important to notice that the inhibitory phase of the response to 5-HT after reserpine was markedly prolonged. Furthermore, a previously ineffective dose of 5-HT (0.5 µg, i.a.) produced a response, a sharp initial increase and subsequent depression of chemoreceptor discharges.

The experiments just described show pronounced effects of ACh, NaCN and 5-HT on chemoreceptor activity after prolonged reserpine-treatment. Qualitatively, there was no appreciable difference between chemoreceptor activation induced by ACh and NaCN before and after reserpine. The results are consistent with those obtained by Zapata *et al.* (1969). However, it is important to notice that the response pattern to 5-HT changed after reserpine, the inhibitory phase of the response being greatly prolonged. This seems to indicate that chemoreceptors may become more sensitive to 5-HT after reserpine.

Effects of 5-hydroxytryptamine on baroreceptor discharges

In order to compare the effects of 5-HT on chemoreceptor discharges with those on other sensory discharges, the baroreceptor discharges frequency was examined after intra-arterial injections of various doses of 5-HT. Nerve filaments containing a single or several units of baroreceptor activity were selected from the carotid sinus nerve. Baroreceptor discharges were identified by: (1) occlusion of the common carotid artery, which caused a decrease or abolition of discharges appearing synchronously with pulsatile pressure of the artery, and (2) making intravenous injections of noradrenaline, which increased the discharge frequency.

Intra-arterial injections of 5-HT (0.5-2 µg) induced an abrupt increase in baroreceptor discharges, as illustrated in Figure 10, in which two single units obtained from different animals were activated by the agent. A burst of discharges occurred about 0.5 s after the injection of 5-HT (2 µg). This excitatory effect on baroreceptor activity lasted for 3 s and thereafter, the spontaneously occurring discharges disappeared. The discharge re-appeared within 20 seconds. The block of discharges which previously occurred synchronously with pulsatile pressure of arterial blood pressure may have been due to a fall in blood pressure caused by 5-HT. However, 20 s after noradrenaline (2 µg/kg, i.v.), when the arterial pressure level was above the control, 5-HT also blocked baroreceptor discharges after inducing an intense increase in discharge frequency. Therefore, the excitatory and inhibitory phase of the response to 5-HT is most probably due to the direct action of 5-HT on the baroreceptor nerve ending.

These observations reveal that the response pattern of chemoreceptor activity to 5-HT is very similar to that of sinus baroreceptors.

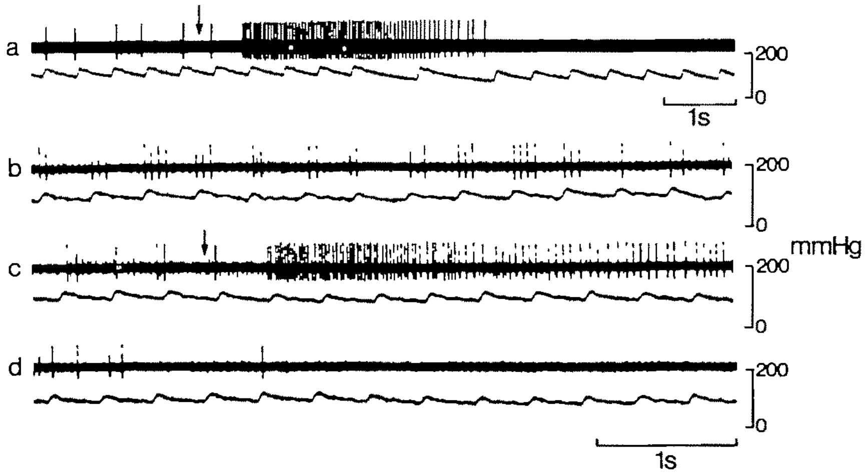

Figure 10 Effect of 5-hydroxytryptamine (5-HT) on baroreceptor activity; (a), (b), (c) and (d) show unitary responses of baroreceptor fibres obtained from different animals to 5-HT (2 μg in 0.25 ml Locke solution, i a.) injected at arrows. (b), (c) and (d) are continuous records. Upper trace in each record shows sinus-baroreceptor fibre discharges. Lower trace, arterial blood pressure.

Discussion

The present experiments have demonstrated that intra-arterial injections of 5-HT induce a sharp and brief increase in chemoreceptor discharges followed by depression or block of naturally occurring discharges. Several experimental observations tend to indicate that the effects of 5-HT on chemoreceptor activity is most probably due to direct actions of 5-HT on the nerve endings of chemoreceptor afferent fibres: (1) relatively high doses of atropine and hexamethonium did not alter the response to 5-HT, indicating that a cholinergic mechanism is not involved in eliciting chemoreceptor activation produced by 5-HT; (2) a burst of chemoreceptor discharges occurred within 0.5 s after the start of the injection of 5-HT. This is characteristic of substances that act directly on sensory nerve endings, while substances that act on 'presynaptic' element, presumably the Type I cell and ischaemia induced by occlusion of the common carotid artery caused a gradual increase in chemoreceptor discharges (Nishi & Eyzaguirre, 1971; Andrew *et al.*, 1972; Eyzaguirre & Nishi, 1974; Nishi, unpublished observation); (3) the latency of the chemoreceptor discharge induced by 5-HT was approximately the same as that of baroreceptor activation produced by the agent, which presumably acts directly on nerve endings. In fact, 5-HT strongly stimulates sensory nerve endings in the rabbit aortic nerves (Douglas & Ritchie, 1957a), in the cat saphenous nerve (Douglas & Ritchie, 1957b; Fjallbrant & Iggo, 1959, 1961), in the rat saphenous nerve (Van Gelder, 1962) and in the hepatic nerve (Andrews, 1968) These considerations are based on the author's conviction that the glomus Type I cells are receptor cells and that the calyx-type endings impinging on them are sensory nerve endings (De Castro, 1951; Eyzaguirre *et al.*, 1972; Hess & Zapata, 1972; Nishi & Stensaas, 1974). However, the possibility that 5-HT may act directly on chemoreceptor free nerve endings in the carotid body (Biscoe, 1971) cannot be ruled out.

With regard to the biphasic response of chemoreceptors to 5-HT, a possible mechanism of action can be offered. The exogenous 5-HT may induce nerve ending depolarization with a rapid onset and, hence, initiate the nerve impulses at the non-myelinated portion of the sensory fibre or at the first node. However, once depolarization reaches a certain level, a cathodal type of block, or desensitization may occur a few seconds after the injection of 5-HT, and depolarization may further develop for another several seconds. When depolarization gradually subsides, the discharges may re-appear. This situation may also occur in the response to 5-HT during asphyxia and hence, induce a further increase in chemoreceptor discharges followed by sudden block of the discharges. Chemoreceptor responses to ACh and NaCN were slightly depressed when these substances were applied within a short period after 5-HT. The mechanism of the depressant action on chemoreceptor activation induced by ACh and NaCN is rather difficult to interpret. However, part of this effect was probably due to sustained depolarization of the nerve endings induced by the previous 5-HT, since in the carotid body superfused with high K^+-solutions which depolarize the receptor elements (nerve endings and/or, presumably, glomus cells), the responses evoked by either ACh or NaCN become smaller than those in the control (Eyzaguirre & Nishi, unpublished observation). A somewhat similar situation can be seen in the cat superior cervical ganglion or other ganglia; 5-HT induces a depolarization with a rapid onset lasting a few seconds and the depolarization produced by small doses is accompanied by asynchronous discharges in the postganglionic nerve (Eccles & Libet, 1961; Herzler, 1961; Gyermek & Bindler, 1961; Jaramillo & Volle, 1968, Watson, 1970), while depression of ganglionic transmission occurs during the sustained depolarization elicited by high doses of 5-HT (Machová & Bőska, 1969).

In the present experiments, repeated injections of 5-HT at short intervals, or a small dose after a large one depressed the responses to 5-HT. The loss of the excitatory effect of 5-HT is probably due to desensitization or tachyphylaxis which is a general characteristic of the action of 5-HT at many peripheral sites, e.g. in the guinea-pig taenia coli preparation (Bülbring & Burnstock, 1960; Born, 1962), or molluscan heart (Welsh, 1957). The present results are also consistent with earlier observations *in vivo* that the chemoreceptor reflex-response to the second dose of 5-HT applied within a period of about 5 min was greatly reduced or absent (Ginzel & Kottegoda, 1954).

With regard to a possible role of 5-HT present in the carotid body, the present experiments with reserpine have not been conclusive, since a quantitative estimate of the degree of depletion of 5-HT by the acute reserpine treatment was not made. However, large doses of reserpine (5-10 mg/kg), which caused a marked decrease in the content of catecholamines in the carotid body (Zapata *et al.*, 1969), may have also reduced the content of 5-HT as well, since in other tissues, acute reserpine-treatment produces considerable decreases in the content of catecholamines as well as 5-HT and furthermore, the time course of the decrease in the 5-HT content is very similar to that for catecholamines (see Carlsson, 1966). Thus, the present results have provided further evidence that 5-HT as well as catecholamines are not main excitatory 'transmitter' substances.

If 5-HT is an excitatory transmitter substance, spontaneously occurring discharges as well as chemoreceptor activation induced by NaCN or by asphyxia should have been depressed or blocked after reserpine-treatment. However, 20 h after a large dose of reserpine, chemoreceptor discharges still occurred, and NaCN as well as asphyxia produced marked chemoreceptor activation (Zapata *et al.*, 1969). Nevertheless, it is possible that 5-HT may play a more subtle role in chemoreceptor mechanisms, since chemoreceptors in the carotid body became more susceptible to 5-HT after reserpine. This may have been due to the denervation sensitization as occurs in other adrenergic structures where reserpine potentiates the response to catecholamines (Bein, 1956; Burn & Rand, 1958; Axelrod, 1964). The endogenous 5-HT could have local modulating effects and either excite or inhibit some receptor components (nerve endings and/or glomus Type I cells), depending upon the amounts of 5-HT released in intimate relationship to appropriate receptors. Another possibility is that endogenous 5-HT could act as a locally circulating hormone influencing the vascular permeability within the carotid body. In fact, 5-HT induces endothelial openings, the endothelial cells becoming partially disconnected along the intercellular junctions (Majno & Palade, 1961; Majno, Schoefl & Palade, 1961). If this is the case in the carotid body capillary, chemoreceptor function would be influenced, since the carotid body consists of networks of capillaries and also the chemoreceptors are very sensitive to changes in local circulation (Joel & Neil, 1963).

The author expresses his sincere gratitude to Dr Eyzaguirre, Department of Physiology, College of Medicine, University of Utah, U.S.A., and to Dr F. Takenaka, Department of Pharmacology, Kumamoto University, Medical School, Japan, for reading the manuscript and commenting on it. This work was supported by a grant, No. 821012, from the Japanese Education Ministry.

Methysergide was kindly supplied by Sandoz Ltd.

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(Received February 10, 1975.
Revised March 30, 1975.)

Br. J. Pharmac. (1975), 55, 41-46

MECHANICAL RESPONSES OF RAT ISOLATED UTERINE HORNS TO TRANSMURAL STIMULATION

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1 Transmural stimulation of rat isolated uterine horns at low pulse width produced contractions. These were antagonized by hyoscine or tetrodotoxin and potentiated by physostigmine.

2 In the presence of hyoscine, and during bradykinin-induced contractions, transmural stimulation produced inhibition. This inhibition was antagonized by guanethidine, propranolol or tetrodotoxin.

3 Hexamethonium or pempidine did not affect responses to transmural stimulation.

4 It is suggested that transmural stimulation is a method of exciting cholinergic motor and noradrenergic inhibitory postganglionic neurones to the rat myometrium.

Introduction

Mechanical responses of uteri from several species to sympathomimetic amines and muscarinic agonists are well characterized. Methods have been described for electrical stimulation of the extrinsic hypogastric nerve supply in the rabbit *in vivo* (Langley & Anderson, 1895; Schofield, 1952) and *in vitro* (Varagić, 1956) and the guinea-pig *in vivo* (Rüsse & Marshall, 1970) with observable mechanical responses. Negligible effects on uterine motility have been seen in the rabbit after electrical stimulation of the pelvic nerve supply *in vivo* (Langley & Anderson, 1895; Schofield, 1952; Bower, 1966). The pelvic and hypogastric nerves are considered to be cholinergic and noradrenergic respectively (Sjoberg, 1967; Marshall, 1970).

In most species both innervations to the myometrium involve 'short' postganglionic neurones arising from ganglia adjacent to the cervix, as section of the extrinsic nerves does not significantly alter myometrial innervation demonstrated histochemically (Sjoberg, 1967; Kanerva, Mustonen & Teräväinen, 1972). Therefore mainly preganglionic neurones would be stimulated by the above methods. Transmural stimulation has been widely used as a method of exciting postganglionic neurones in other tissues. A method of transmural stimulation of isolated uterine horns of the rat producing cholinergic-mediated contractions and noradrenergic-mediated inhibitions is described.

Methods

Virgin Sprague-Dawley rats, 175 to 250 g, were ovariectomized and, 2 to 4 weeks later, injected subcutaneously with 17β-oestradiol (1.8×10^{-8} mol/kg) in arachis oil daily for 7 days and killed on day 8.

The isolated uterine horns were mounted in a tissue bath containing Krebs solution (mM: Na^+ 143.5, K^+ 5.94, Ca^{++} 2.55, Mg^{++} 1.19, Cl^- 128.4, HCO_3^- 25.0, $SO_4^=$ 1.19, $H_2PO_4^-$ 1.19, glucose 11.1) at 32°C under a resting tension of 0.5 gram. Tension changes were recorded isometrically. Isolated uterine horns under these conditions show minimal spontaneous mechanical activity (Hollingsworth, 1974).

A Grass S8 stimulator was used to stimulate the tissue transmurally via a pair of parallel stainless steel wire electrodes, 0.5 cm apart. The intraluminal electrode was positive. Pulses lasting 0.5 ms and of supra-maximal voltage (40 V) were applied.

Contractile responses to transmural stimulation

After obtaining constant contractions to acetylcholine (1×10^{-6} mol/l), an ascending frequency-effect curve was determined by stimulating transmurally, using 10 s trains applied every 100 seconds. Responses were obtained to acetylcholine and other drugs, applied for a

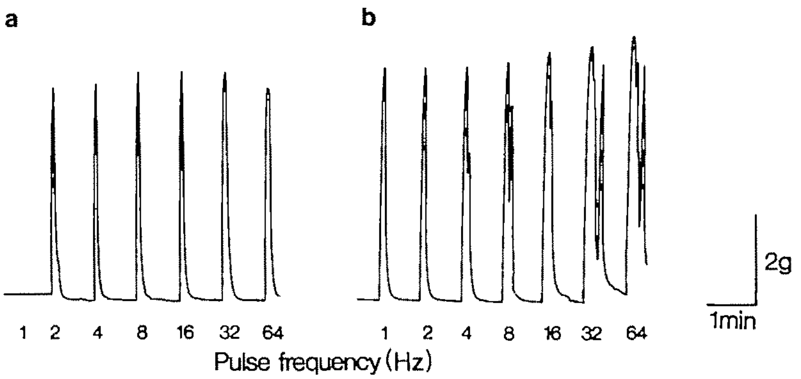

Figure 1 (a) Contractions of an isolated uterine horn of the rat produced by transmural stimulation in Krebs solution and (b) in the presence of physostigmine (1×10^{-5} mol/l). Stimulation parameters: 0.5 ms pulses; 40 V, supra-maximal voltage; 10 s trains applied every 100 seconds.

contact time of 30 s (or 45 s for bradykinin) every 3 minutes.

Inhibitory responses to transmural stimulation

For these experiments the Krebs solution contained hyoscine (1×10^{-8} mol/l). Repeated sustained contractions of the rat uterine horns were achieved by adding a standard concentration of bradykinin (either 9.4×10^{-9} or 4.7×10^{-8} mol/l) for 2 min every 5 minutes. This concentration of bradykinin produced a contraction which was 90% of the maximal contraction to acetylcholine. One min after the addition of the bradykinin, the tissue was stimulated transmurally at a pulse frequency of 2 Hz for 10 seconds. A frequency-effect curve was obtained by repeating the bradykinin standard and using higher frequencies. Concentration-effect curves to noradrenaline and aminophylline were determined in a similar manner by adding them 40 s after the addition of bradykinin.

Modification of responses to transmural stimulation and drugs

Control responses were obtained to transmural stimulation and drugs. Responses were then re-examined, after 30 min incubation, either in Krebs solution (controls) or in Krebs solution containing the modifying drug (test).

Contractile responses to transmural stimulation and drugs were measured as pen deflections (mm). Inhibitory responses were measured as percentage inhibition of bradykinin contractions. These were converted to percentages of the maximal control responses either to acetylcholine (contraction experiments) or to noradrenaline (inhibition experiments). Potencies of drugs are expressed as the mean negative log EC_{50} ± s.e. mean. Horizontal shifts of the EC_{50}'s were used to determine the extent of antagonism or potentiation.

The Mann-Whitney U-test or the Wilcoxon matched pair signed rank test (Siegel, 1956) were used to test the significance of differences.

Drugs

The following drugs were used: acetylcholine chloride (Lematte et Boinot); aminophylline (United Chemists Association); bradykinin (Sandoz); guanethidine sulphate (CIBA); hexamethonium bromide (May & Baker); 5-hydroxytryptamine creatine sulphate (Koch-Light); hyoscine hydrobromide (Koch-Light); nicotine hydrogen tartrate (BDH); 17β-oestradiol (BDH); pempidine tartrate (May & Baker); physostigmine salicylate (BDH); propranolol hydrochloride (I.C.I.) and tetrodotoxin (Sankyo).

Results

Contractile responses to transmural stimulation

Transmural stimulation of the isolated uterine horns produced single phasic contractions with a latency and duration of approximately 5 s and 30 s respectively (Figure 1). Contractions were more consistently obtained with a pulse duration of 0.5 ms than with one of 0.2 ms. The lowest frequency at which the virtually all-or-none contractions were obtained varied from 1 to 4 Hz in different preparations, accounting for the

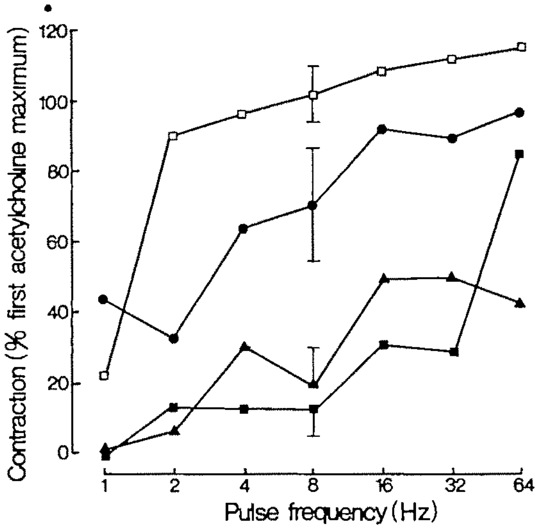

Figure 2 Contractile responses of isolated uterine horns of rat to transmural stimulation in controls (●; $n = 10$) or in the presence of physostigmine (□; 1×10^{-5} mol/l; $n = 8$), hyoscine (▲; 1×10^{-8} mol/l; $n = 9$) or tetrodotoxin (■; 3.1×10^{-7} mol/l; $n = 7$). Stimulation parameters. 0.5 ms pulses, 40 V, 10 s trains every 100 seconds. Means are shown and representative s.e.'s indicated. The means for physostigmine at 2 Hz, for hyoscine at 16 and 64 Hz and for tetrodotoxin at 4, 8, 16 and 32 Hz differed ($P < 0.05$) from the corresponding control means.

frequency-effect curves in Figure 2. The maximal tension to transmural stimulation was 88% of the maximum response to acetylcholine. At 37°C, when spontaneous contractions were seen, drugs and transmural stimulation induced multi-phasic contractions.

Hyoscine (1×10^{-8}mol/l) and tetrodotoxin (3.1×10^{-7} mol/l) greatly reduced the responses to transmural stimulation ($P < 0.05$) at most frequencies (Figure 2). Physostigmine (1×10^{-5}mol/l) increased both the duration (Figure 1) and the maximal response to transmural stimulation at most frequencies (Figure 2). Repeat control responses to transmural stimulation did not differ from the initial control responses.

The concentration-effect curves with acetylcholine were shifted to the right 31.6-fold by hyoscine ($P < 0.01$) and shifted to the left 2.1-fold by physostigmine ($P < 0.05$). Repeat control concentration-effect curves to acetylcholine, 5-hydroxytryptamine and bradykinin and responses to 5-hydroxytryptamine and bradykinin in the presence of hyoscine did not differ from the initial control responses.

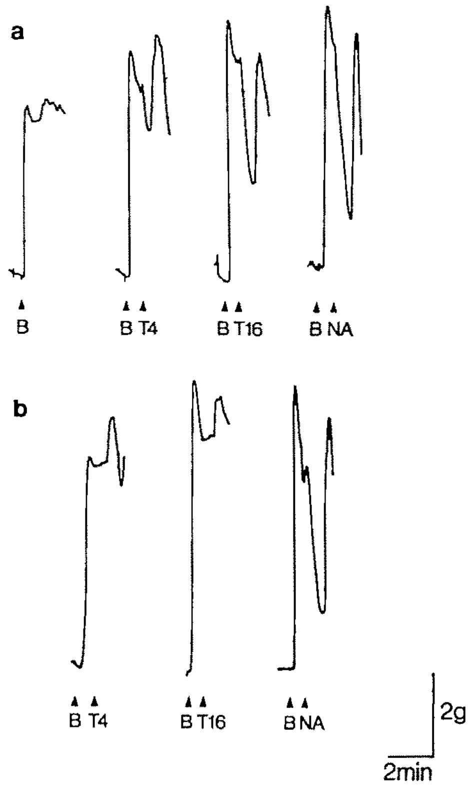

Figure 3 Inhibitory responses of an isolated uterine horn of rat to transmural stimulation (T4 = 4 Hz; T16 = 16 Hz) and to noradrenaline (NA; 5×10^{-7} mol/l), (a) in Krebs solution and (b) in the presence of guanethidine (5×10^{-6} mol/l). Bradykinin (B, 9.4×10^{-9} mol/l) was added for 2 min every 5 min. Stimulation parameters· 0.5 ms pulses; 40 V, supra-maximal voltage; 10 s trains. The Krebs solution contained hyoscine (1×10^{-8} mol/l).

Hexamethonium (1×10^{-5}mol/l) had no effect on contractions to transmural stimulation or acetylcholine ($n = 3$). Hexamethonium (1×10^{-4} mol/l) produced a 50% reduction in maximal reponses to transmural stimulation and to acetylcholine.

Inhibitory responses to transmural stimulation

Before inhibitory responses of the rat isolated uterine horns could be observed tone had to be induced. Among the contractile agents tested (acetylcholine, 5-hydroxytryptamine, oxytocin, angiotensin and bradykinin), only bradykinin induced a sustained tone for more than 1 min (Figure 3). Even with bradykinin, there were small

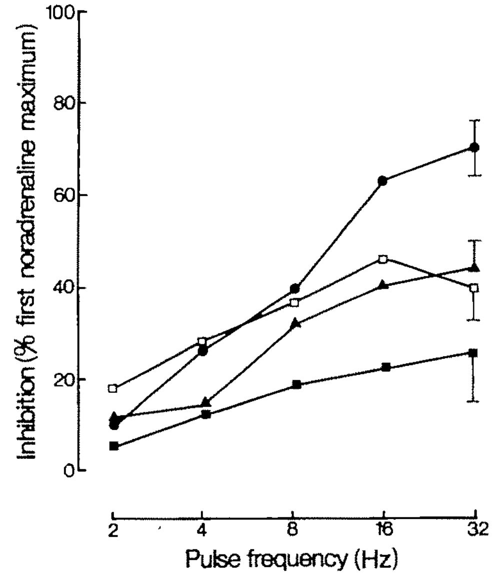

Figure 4 Inhibitory responses of rat isolated uterine horns to transmural stimulation in controls (●; $n = 10$) or in the presence of propranolol (□; 1×10^{-8} mol/l; $n = 8$), guanethidine (▲; 5×10^{-6} mol/l; $n = 10$) or tetrodotoxin (■; 3.1×10^{-7} mol/l; $n = 9$). Transmural stimulation (0.5 ms pulses, 40 V, 10 s trains) was performed during a bradykinin-induced contraction. Results are means and representative s.e.'s are indicated. The means for all test curves differed ($P < 0.05$) from the controls at 16 and 32 Hz.

fluctuations in the sustained tone (17 ± 2% in controls, $n = 43$; 8 ± 3% in repeated controls, $n = 27$; measured as a percentage of the first noradrenaline maximum) which have been called spontaneous inhibitions. There was no marked tachyphylaxis in the contractile response to bradykinin when added to the tissue for 2 min every 5 min over a 3 h period.

Transmural stimulation, during a contraction induced by bradykinin, and in the presence of hyoscine (1×10^{-8} mol/l), induced inhibitions after a latency of 5 to 10 s (Figure 3). The inhibitions tended to be multiphasic. The magnitude of the inhibitions were proportional to pulse frequency up to 32 Hz (Figure 4). The response at this frequency was 71% of the maximum inhibition produced by noradrenaline and a 51% inhibition of the bradykinin contraction. At higher pulse frequencies an increase in tone was often seen preceding the inhibition. Repeat control responses to transmural stimulation did not differ from the initial control responses. Both noradrenaline (2×10^{-8} to 3.1×10^{-4} mol/l; Figure 3) and aminophylline (2.5×10^{-4} to 4×10^{-3} mol/l) produced concentration-related inhibitions of bradykinin induced contractions.

Inhibitory responses to transmural stimulation were greatly reduced by tetrodotoxin (3.1×10^{-7} mol/l, $P < 0.05$), and to a lesser extent by propranolol (1×10^{-8} mol/l, $P < 0.05$) or guanethidine (5×10^{-6} mol/l, $P < 0.05$) (Figures 3 and 4). In two experiments nicotine (2×10^{-8} to 6.25×10^{-5} mol/l) produced small and inconsistent inhibitions of bradykinin-induced contractions. In two further experiments pempidine (1×10^{-5} mol/l) did not modify these responses or the responses to transmural stimulation.

The specificity of the modifying agents was determined by any shifts they produced in the concentration-effect curves to noradrenaline and aminophylline (Table 1). Propranolol

Table 1 Potencies, as mean negative log molar EC_{50}'s, of noradrenaline and aminophylline in inhibiting bradykinin-induced contractions of isolated uterine horns of rat in controls, or after 30 min incubation with a modifying agent

Modifying agent		*Noradrenaline*	*Aminophylline*
None	Control	6.72 ± 0.13 (11)	3.09 ± 0.07 (7)
	Repeat control	6.70 ± 0.19 (11)	3.04 ± 0.12 (6)
Propranolol (1×10^{-8} mol/l)	Control	6.88 ± 0.04 (6)	3.26 ± 0.07 (5)
	Test	6.18 ± 0.16 (8)*	3.40 ± 0.07 (4)
Guanethidine (5×10^{-6} mol/l)	Control	6.79 ± 0.22 (10)	3.30 ± 0.06 (9)
	Test	6.96 ± 0.23 (7)	3.39 ± 0.07 (6)
Tetrodotoxin (3.1×10^{-7} mol/l)	Control	7.00 ± 0.10 (9)	–
	Test	6.80 ± 0.20 (7)	

Results are means with s.e. mean. Number of values in parentheses.

* Significant change of $-\log EC_{50}$ ($P < 0.05$, Wilcoxon matched pair signed rank test).

(1×10^{-8} mol/l) produced a 6-fold shift to the right of the concentration-effect curve to noradrenaline without affecting responses to aminophylline. This concentration of propranolol abolished the spontaneous inhibitions; higher concentrations also reduced bradykinin contractions. Guanethidine and tetrodotoxin did not alter the mean $-\log$ EC_{50}'s to noradrenaline or aminophylline.

Discussion

Electrical stimulation at low pulse width has been widely used as a method of eliciting neurotransmitter release in a variety of tissues. The method has been little used with uterine tissues and then only for producing contractile responses. Paterson (1965) has described transmural stimulation of isolated uterine horns of the rat without analysis of the responses. Nakanishi, McLean, Wood & Burnstock (1969) and Nakanishi & Wood (1971) have suggested that contractile responses of isolated strips of human uterus to transmural stimulation had both noradrenergic and cholinergic components, although contractions were little affected by atropine. The contractile and inhibitory responses obtained here to transmural stimulation of rat uterine horns were antagonized by tetrodotoxin, suggesting that they were nerve-mediated.

The contractile responses to transmural stimulation at low pulse width were antagonized by hyoscine and potentiated by physostigmine. This, plus similar results using a perfused uterine horn preparation (Hollingsworth, 1974), where contractions of circular smooth muscle were probably being measured, suggests that both circular and longitudinal smooth muscle receive a cholinergic motor innervation. Histochemical findings support this (Adham & Schenk, 1969; Hervonen, Kanerva & Lietzén, 1973; Hollingsworth, 1974). Electrical stimulation of the pelvic parasympathetic nerves in the rabbit *in vivo* only occasionally produced uterine horn contractions (Schofield, 1952) and did not induce action potentials in uterine nerves (Bower, 1966). The effects seen here in the rat but not in the rabbit might be a species difference or might be related to the oestrogen pre-treatment used in this work which increased acetylcholinesterase staining, typical of cholinergic nerves, suggesting an increased density of innervation (Hervonen *et al.*, 1973; Hollingsworth, unpublished observations).

Physostigmine produced potentiation of contractile responses to both transmural stimulation and exogenous acetylcholine. It is therefore likely that cholinesterases of the rat uterine horns that have been demonstrated biochemically (Foley & McPhillips, 1973) and histochemically, limit the effect of released neurotransmitter.

The method developed here allows the quantitative study of inhibitory responses to transmural stimulation, or exogenous drugs, in uterine horns which do not exhibit an inherent sustained tone. Guanethidine and propranolol antagonize the inhibitory responses to transmural stimulation and this suggests a noradrenergic innervation. Both inhibitory and contractile uterine horn responses to hypogastric nerve stimulation have been described in the rat, depending on the animal's hormonal state (Labate, 1941; Butterworth & Randall, 1970). It is possible that the inhibitory responses to transmural stimulation were due to adrenaline release as rat uteri have a high adrenaline content (Wurtman, Chu & Axelrod, 1963). Collection and assay methods are necessary to prove whether the released transmitter is noradrenaline or adrenaline.

Only a sparse noradrenergic innervation of the rat myometrium has been described histochemically (Hervonen *et al.*, 1973; Hollingsworth, 1974). This is supported by the absence of potentiation of noradrenaline responses by guanethidine here compared to tissues containing a dense noradrenergic innervation, where potentiation is seen. However, the mechanical activity of the rat uterus is readily affected by hypogastric nerve stimulation (Labate, 1941, Butterworth & Randall, 1970) and transmural stimulation. Mechanical responses to transmural stimulation were seen at the same pulse frequencies as the frequencies of action potential discharge recorded by Bower (1966) in rabbit uterine nerves. This suggests a rôle for the autonomic innervation in regulating uterine motility. Rüsse & Marshall (1970) have suggested from *in vivo* studies that one function of the uterine noradrenergic innervation of the guinea-pig is to modulate responses to contractile agents. Use of the present *in vitro* method will allow the study of the mechanism of any modulating action.

The failure of hexamethonium and pempidine to modify responses to transmural stimulation suggests that predominantly postganglionic neurones are being stimulated by this technique. This agrees with histochemical evidence that the majority of noradrenergic and cholinergic postganglionic neurones to the myometrium have their synapses in the paracervical ganglion adjacent to the cervix (Sjoberg, 1967; Mustonen & Terävainen, 1971; Kanerva *et al.*, 1972). It has been suggested that these 'short' noradrenergic neurones, which are confined to the urogenital tract, display functional differences from 'long' noradrenergic neurones. They are largely unaffected by postnatal administration of nerve growth factor antiserum, they are resistant to the effects of reserpine and 6-hydroxydopamine, they are difficult to deplete by nerve stimulation and the sex steroids can modify the transmitter content of the tissue (Owman & Sjoberg, 1973). This *in vitro* technique could be used to see if there are other functional differences.

Failure of the blocking drugs to abolish completely the contractile and inhibitory responses to transmural stimulation could be interpreted in two ways. Firstly, the concentrations used were inadequate to abolish the actions of released acetylcholine and noradrenaline, or secondly, that transmural stimulation elicited the release of some other transmitter.

This work was supported by the Lalor Foundation, U.S.A. The author wishes to acknowledge the excellent technical assistance of Mrs C. Isherwood and Miss J. Brooks. Thanks are due to Sandoz Products Ltd. for a gift of synthetic bradykinin.

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(Received March 14, 1975.
Revised April 14, 1975.)

Br. J. Pharmac., (1975), 55, 47-49

THE EFFECTS OF PROSTAGLANDINS E_1, E_2 AND $F_{2\alpha}$ ON VAGAL BRADYCARDIA IN THE ANAESTHETIZED MOUSE

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1 In anaesthetized mice prostaglandins E_1 and E_2 reduced the bradycardia caused by electrical stimulation of the sectioned peripheral vagus nerve; prostaglandin $F_{2\alpha}$, produced only a slight inhibition of the vagal response.

2 None of the prostaglandins studied affected acetylcholine-induced bradycardia.

3 Prostaglandins modify parasympathetic nerve activity *in vivo* presumably by a pre-synaptic action.

Introduction

Certain criteria must be satisfied for prostaglandins to modulate the release of autonomic neurotransmitter substances by an endogenous feedback mechanism.

In recent years, several reports have shown that prostaglandins of the E series may reduce the responses of isolated cardiac preparations to both sympathetic (Hedqvist, Stjarne & Wennmalm, 1970; Hedqvist & Wennmalm, 1971) and parasympathetic nerve stimulation (Wennmalm & Hedqvist, 1971; Hadhazy, Illés & Knoll, 1973). This evidence, together with reports that prostaglandin-like substances may be released upon sympathetic (Sammuelsson & Wennmalm, 1971) and also upon parasympathetic nerve stimulation (Coceani, Pace-Asciak, Volta & Wolfe, 1967; Junstad & Wennmalm, 1974), indicates a possible involvement of prostaglandins in the transmission process.

The aims of the current studies were to see whether certain prostaglandins were capable of inhibiting the effects of cardiac vagal stimulation in anaesthetized mice.

Methods

Male mice of Tuck No. 1 strain, weighing 25-40 g were anaesthetized with a mixture of chloralose (100 mg/kg) and hexobarbitone (100 mg/kg) administered via an indwelling cannula in the caudal vein which also served for all subsequent injections. The animals were artificially ventilated at 90 strokes/min, with a volume of approximately 0.5 ml. Blood pressure and integrated heart rate were measured from the cannulated left common carotid artery. On each side of the neck the cervical vagus and sympathetic nerves (Large, 1975) were separated and sectioned; bipolar platinum electrodes were used to stimulate the cardiac end of the right or left vagus with rectangular pulses of 0.5 ms duration at supramaximal voltage (6-12V) with liquid paraffin for insulation. The initial mean arterial blood pressure was 44 ± 2 mmHg (mean ± s.e.) and the initial heart rate 455 ± 11 beats/minute.

The vagus nerve was initially stimulated with 1 Hz and the frequency progressively changed through 2, 5, 10, 20 to 50 Hz as the heart rate stabilized at each frequency. The blood pressure and heart rate having returned to resting values, the vagus nerve was then stimulated intermittently with a selected frequency every 3 min using a constant number of shocks (50, 100 or 200). Following two consecutive control responses, S_1 and S_2, an intravenous injection of prostaglandin E_1, E_2 or $F_{2\alpha}$ was made 10-20 s prior to obtaining the third response, S_3. The bradycardia induced by vagal stimulation in the presence of the prostaglandin was compared with that in its absence by the change in the ratios, S_2/S_1 and S_3/S_2. Differences between the ratios S_2/S_1 and S_3/S_2 were analysed by Student's *t*-test.

A similar procedure was adopted to investigate the effects of the prostaglandins on the bradycardia induced by intravenous acetylcholine but, in order to obtain bradycardia, physostigmine had to be given beforehand.

Results

Prostaglandins E_1 and E_2 (in doses from 2.5 to 20 μg/kg) produced a fall in both systolic and diastolic pressures, the magnitude of which was dependent upon the resting blood pressure. In most experiments heart rate was unaffected though occasionally a tachycardia not exceeding 10 beats/min was seen. By contrast, prostaglandin $F_{2\alpha}$ (10 or 20 µg/kg) produced a rise in both systolic and diastolic pressures, with little change in the resting heart rate.

The bradycardia induced by stimulating the cardiac end of the cut vagus nerve could be inhibited by the prior administration of prostaglandin E_1 or E_2. This inhibitory effect was of short duration, responses returning to control levels usually within a 6 min period. A slight inhibition of vagal bradycardia was seen with prostaglandin $F_{2\alpha}$ although no differences were observed between the two dose levels employed. More experiments are needed to determine if larger doses of prostaglandin $F_{2\alpha}$ could produce further inhibition. Some of these results are illustrated in Table 1. The effect of the E prostaglandins was, however, dose-dependent in that progressively greater inhibition occurred as the dose was raised. A statistical analysis has only been possible so far on the results obtained with 10 µg/kg prostaglandin E_1 and E_2.

Acetylcholine (2-5 µg/kg) produced only vasodepression but in the presence of physostigmine salicylate (100 µg/kg) it caused a larger fall in blood pressure and also a bradycardia of 92 ± 6 beats/minute. As may be seen from Table 1 this bradycardia was unaffected by any of the prostaglandins. Physostigmine also enhanced the responses to vagal stimulation but did not modify the inhibitory effects of prostaglandin E_1 or E_2.

Since the E prostaglandins were more potent than prostaglandin $F_{2\alpha}$ in reducing the vagal bradycardia, experiments were carried out to determine whether the inhibition was consequent upon the fall in blood pressure produced by the E prostaglandins. Neither bradykinin (0.5-5 µg/kg), which caused a prolonged fall in blood pressure, nor acetylcholine (2-5 µg/kg) modified the response to vagal stimulation.

Discussion

These results demonstrate that prostaglandins E_1, E_2 and, to a lesser extent, $F_{2\alpha}$ can reduce the responses of the heart *in vivo* to parasympathetic nerve stimulation. These inhibitory effects appear to be presynaptic in nature.

Since the acetylcholine-induced bradycardia was unaffected by any of the prostaglandins it is tempting to conclude that these substances were preventing the release of acetylcholine from the parasympathetic nerve endings, though an effect on ganglionic transmission cannot be excluded. Vasodepression as such was not responsible for the inhibitory effect of the E prostaglandins since neither bradykinin nor acetylcholine were capable of reducing the responses to vagal stimulation. Furthermore larger doses of E prostaglandins, which did not always produce a greater fall in blood pressure, caused a greater inhibition of the bradycardia.

Benz & Salzmann (1974) stated that prostaglandins did not modify vagal activity in the anaesthetized cat, despite observing a small

Table 1 Effect of intravenous prostaglandins (PGs) on the bradycardia induced in anaesthetized mice either by electrical stimulation of the vagus nerve or by acetylcholine

	Control			*PGE_1*		*PGE_2*		*$PGF_{2\alpha}$*	
	Mean ΔHR	S_2/S_1 †	n	*(10 µg/kg)* S_3/S_2	n	*(10 µg/kg)* S_3/S_2	n	*(10 or 20 µg/kg)* S_3/S_2	n
‡ Acetylcholine 2 to 5 µg/kg	92	0.97±0.02	12	0.99±0.04	6	0.97±0.09	3	1.02±0.04	3
10 Hz 5 s	88	0.97±0.02	12	0.42±0.09***	4	0.46±0.05***	4	0.95±0.03	4
10 Hz 10 s	124	1.03±0.02	20	0.38±0.12***	6	0.36±0.06***	8	0.92±0.03**	6
20 Hz 5 s	125	1.05±0.01	7	0.53±0.13***	3	0.54±0.08***	4	–	
20 Hz 10 s	137	0.99±0.04	8	0.55±0.16**	3	0.39±0.11***	5	–	
50 Hz 2 s	272	1.04±0.03	5	0.25	2	0.62±0.13*	3	–	

All values are means ± s.e mean. † S_1 and S_2 are two successive control responses just prior to S_3 which is the response obtained 10 to 20 s after injection of the prostaglandin. ‡ Responses to acetylcholine were obtained after the administration of physostigmine salicylate (100 µg/kg) both drugs being given intravenously.

Comparison of ratio S_2/S_1 with appropriate S_3/S_2; Student's *t* test, *P* values· * < 0.05 > 0.01; ** < 0.01 > 0.001; *** < 0.001. There were no statistically significant differences between any pairs of S_3/S_2 values for a given prostaglandin during the periods of nerve stimulation.

reduction in the magnitude of the vagal bradycardia with prostaglandins E_1 or $F_{2\alpha}$, whilst E_2 was without activity. Although species differences may exist, a direct comparison of results is difficult, since in their experiments the prostaglandins were given by intravenous infusion at rates from 0.001 to 1.0 μg/minute.

Our results suggest that prostaglandins, particularly of the E series, may modulate the release of the parasympathetic neurotransmitter and demonstrate that inhibitory effects can occur *in vivo*.

At present we are studying the effects of prostaglandin-synthetase inhibitors on this phenomenon and also the actions of prostaglandins on sympathetic function in mice.

W.F. is an MRC scholar. The prostaglandins were kindly provided by Dr J.E. Pike of the Upjohn Company, Kalamazoo, U.S.A.

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(Received April 4, 1975.
Revised June 12, 1975.)

Br. J. Pharmac. (1975), 55, 51-56

ON THE ABILITY OF PROSTAGLANDIN E_1 AND ARACHIDONIC ACID TO MODULATE EXPERIMENTALLY INDUCED OEDEMA IN THE RAT PAW

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1 Prostaglandins E_1 and E_2 but not prostaglandin $F_{2\alpha}$, arachidonic acid or linolenic acid, produced slight oedema when injected into the rat hindpaw.

2 Prostaglandin E_1 potentiated hindpaw oedema produced by carrageenan, kaolin, bradykinin and trypsin but not that produced by 5-hydroxytryptamine (5-HT), histamine, dextran B or compound 48/80. Carrageenan- and bradykinin-induced paw oedemas were also potentiated by prostaglandin E_2. Arachidonic acid potentiated responses to carrageenan and kaolin but not responses to bradykinin, trypsin, 5-HT, histamine, dextran B or compound 48/80. Linolenic acid did not potentiate hindpaw oedema induced by carrageenan.

3 Potentiation of carrageenan-induced oedema by prostaglandin E_1 was not diminished by pretreatment with indomethacin, hydrocortisone or cyproheptadine. However, arachidonic acid potentiation of carrageenan oedema was reduced by pretreatment with non-steroidal anti-inflammatory drugs but not by anti-inflammatory steroids or by paracetamol.

4 The enhancement of the response to carrageenan and kaolin by prostaglandins E_1, E_2 and arachidonic acid is discussed in terms of kinin mediation.

Introduction

It has been proposed that prostaglandins are mediators of the inflammatory response (Vane, 1972). Indeed, the *in vivo* potency of non-steroidal anti-inflammatory drugs has been correlated with their ability to inhibit *in vitro* the synthesis of prostaglandins by enzyme preparations from several tissues including dog spleen (Flower, Gryglewski, Herbaczynska-Cedro & Vane, 1972) and sheep seminal vesicles (Ham, Cirillo, Zanetti, Shen & Kuehl, 1972). Non-steroidal anti-inflammatory drugs reduce prostaglandin E levels in carrageenan-induced inflammatory exudates (Willis, Davison, Ramwell, Brocklehurst & Smith, 1972). Furthermore, prostaglandins E_1 and E_2 increase vascular permeability when injected intradermally in rats (Arora, Lahiri & Sanyal, 1970; Kaley & Weiner, 1971) and produce a weal and flare reaction when injected intradermally in man (Crunkhorn & Willis, 1971).

The administration of nanogram quantities of prostaglandin E_1 into the rat hindpaw produces only slight oedema (Arora *et al.*, 1970). The response is not dose-related, for at higher concentrations (40-80 μg), prostaglandin E_1 does not cause inflammation (Glenn, Bowman & Rohloff, 1972). However, prostaglandins may play a modulatory role in inflammation. For example, Ferreira (1972) reported that prostaglandin E_1 potentiates the pain producing actions of intradermal injections of bradykinin and histamine in man. Furthermore, prostaglandins E_1 and E_2 potentiate carrageenan-induced paw oedema in the rat (Moncada, Ferreira & Vane, 1973; Ferreira, Moncada, Parsons & Vane, 1974). In order to determine whether prostaglandins modulate other oedemas, we have examined the effects of prostaglandins E_1, E_2 and $F_{2\alpha}$ and of a prostaglandin precursor, arachidonic acid, upon rat paw oedema induced by various phlogogenic agents. A preliminary report of part of this work has been published (Lewis, Nelson & Sugrue, 1974).

Methods

Male Wistar rats (CE/CFHB, Carworth Europe) weighing 80-100 g were used in groups of 7-10 animals in all experiments. Phlogogens, dissolved or suspended in 0.1 ml 0.9% w/v NaCl solution (saline), were injected into the subplantar region of the right hindpaw. The left hindpaw received an equal volume of saline. Phlogogens were injected

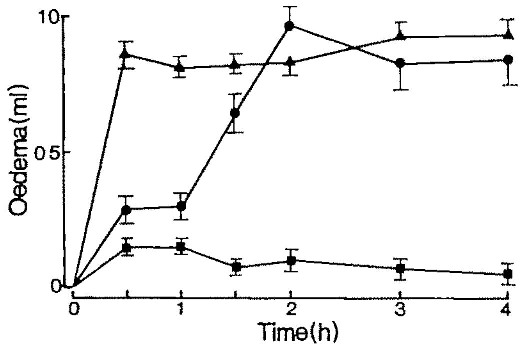

Figure 1. Increase in paw volume following subplantar injection of carrageenan (1 mg) (●), prostaglandin E_1 (0.1 μg) (■), or a combination of carrageenan (1 mg) and prostaglandin E_1 (0.1 μg) (▲). Each value is the mean ± s.e. mean of 7-10 observations. Potentiation of the carrageenan response by prostaglangin E_1 was significant ($P < 0.001$) at 0.5 h and 1.0 hour.

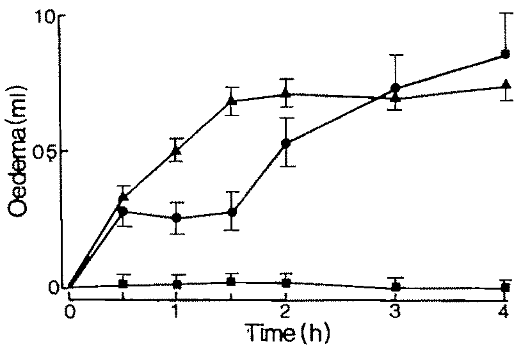

Figure 2. Increase in paw volume following subplantar injection of carrageenan (1 mg) (●), arachidonic acid (100 μg) (■) or a combination of carrageenan (1 mg) and arachidonic acid (100 μg) (▲). Each value is the mean ± s.e. mean of 7-10 observations. Potentiation of the response to carrageenan by arachidonic acid was significant at 1.0 h ($P < 0.01$) and 1.5 h ($P < 0.001$).

either alone or in combination with prostaglandins E_1, E_2, $F_{2\alpha}$, arachidonic acid or linolenic acid. Other groups of rats received either prostaglandin or fatty acid alone.

Anti-inflammatory agents were injected subcutaneously in propan-1, 2-diol (1.0 ml/kg) 1 h before induction of paw oedema. Preliminary experiments revealed that the subcutaneous injection of this volume of propan-1, 2-diol had no effect upon oedema induced in the rat paw by the phlogogens under study.

The volumes of both hindpaws of each rat were measured by mercury displacement plethysmometry at 0, 0.5, 1, 1.5, 2, 3 and 4 h after induction of paw oedema. Mean differences between right and left hindpaw volumes for each group were calculated for each time interval. The statistical significance of differences between treatments was determined by Student's *t* test (2-tailed).

Drugs

Arachidonic acid and linolenic acid (Grade 1, Sigma) were diluted with ethanol to give 40 mg/ml solutions. Aliquots (0.5 ml) were then sealed in glass vials under nitrogen and stored at −15°C until required. The ethanolic solutions were withdrawn from the vials and further dilutions made with saline immediately before use. Prostaglandins E_1, E_2 and $F_{2\alpha}$ were stored at −15° C as ethanolic solutions (10 mg/ml) in rubber capped bottles and aliquots withdrawn through a hypodermic needle and diluted with saline immediately before use.

Other drugs used in this study were: dextran B (150,000-200,000 mol. wt.), kaolin (light grade) and 4-acetamidophenol (paracetamol) (BDH), bradykinin BRS 690 (Sandoz), carrageenan (Viscarin, Marine Colloids), compound 48/80 (Wellcome), cyproheptadine hydrochloride and indomethacin (Merck, Sharp & Dohme), fluocinolone acetonide (ICI), histamine diphosphate, 5-hydroxytryptamine (serotonin) creatinine sulphate and trypsin (Koch-Light), hydrocortisone acetate (Organon), naproxen (Syntex) and phenylbutazone (Geigy). Where applicable doses refer to the appropriate salt.

Results

Interactions of prostaglandins or unsaturated fatty acids with carrageenan

Prostaglandin E_1 (0.1 μg) induced a mild paw swelling upon subplantar injection (Figure 1), maximum oedema (0.15-0.25 ml) being observed between 0.5 h and 1 hour. However, within 0.5 h of the administration of prostaglandin E_1 in combination with a submaximal dose of carrageenan (1 mg), a marked oedema had developed. The potentiation of carrageenan-induced oedema by prostaglandin E_1 (0.1 μg) was significant at 0.5 and 1 h ($P < 0.001$). Prostaglandin E_2 (0.1 μg) also caused mild oedema for up to 1 h after injection (0.13 ± 0.03 ml). Furthermore, oedema induced in the rat paw by carrageenan (1 mg; 0.33 ± 0.04 ml) was significantly potentiated at 1 h ($P < 0.01$) by the concomitant administration of prostaglandin E_2 (0.1 μg; 0.64 ± 0.06 ml). In contrast to prostaglandins E_1 and E_2, $F_{2\alpha}$ (0.25 μg) did not elicit any oedema and did not enhance oedema induced by carrageenan (1 mg).

Subplantar injection of either arachidonic acid (100 μg) (Figure 2) or linolenic acid (100 μg) failed to elicit an inflammatory response. However, arachidonic acid (100 μg) potentiated the effect of carrageenan (1 mg), the oedema induced by administration of the combination being significantly greater than the additive effect at 1 h ($P < 0.01$) and 1.5 h ($P < 0.001$). In contrast, carrageenan-induced oedema was not altered by the concomitant administration of linolenic acid (100 μg).

The difference in the response to carrageenan (cf. Figures 1 and 2) is to be noted. It confirms reports that this response varies in different rats and on different days (Green, Green, Murray & Wilson, 1971; Crunkhorn & Meacock, 1972).

Table 1. Effect of drugs upon potentiation of carrageenan-induced paw oedema by arachidonic acid

	Dose (mg/kg)	*% reduction of carrageenan oedema*	*% reduction of potentiation*
Indomethacin	5	52.0 ± 8.2	52.2 ± 5.7
Naproxen	30	35.2 ± 11.9	47.6 ± 3.7
Phenylbutazone	75	30.7 ± 8.6	63.6 ± 3.1
Fluocinolone	1	40.8 ± 11.8	4.1 ± 4.0
Hydrocortisone	100	34.7 ± 9.1	3.7 ± 3.9
Paracetamol	200	61.9 ± 12.8	10.5 ± 5.9

Drugs were administered subcutaneously 0.5 h before, and paw volumes measured 1.5 h after subplantar injection of either carrageenan (1 mg) or carrageenan (1 mg) plus arachidonic acid (100 μg). Each result is the mean ± s.e. mean from 7-10 animals.

Effects of drugs upon prostaglandin E_1 and arachidonic acid-carrageenan interactions

Doses of drugs were chosen which inhibit by 30-60% the paw oedema developing 0.5-2 h after carrageenan administration. Indomethacin (5 mg/kg), hydrocortisone (100 mg/kg) or cyproheptadine (20 mg/kg) given subcutaneously 0.5 h before the combination of prostaglandin E_1 (0.1 μg) and carrageenan (1 mg), did not modify the resulting oedema at +0.5 hour. However, indomethacin (5 mg/kg), naproxen (30 mg/kg) and phenylbutazone (75 mg/kg) did reduce the archidonic acid (100 μg) potentiation of carrageenan-induced oedema at +1.5 h, whilst fluocinolone (1 mg/kg), hydrocortisone (100 mg/kg) and paracetamol (200 mg/kg) did not (Table 1).

Effects of prostaglandin E_1 and arachidonic acid upon rat paw oedema induced by various phlogogenic agents

Both prostaglandin E_1 (0.1 μg; Table 2) and arachidonic acid (100 μg; Table 3) potentiated oedema of the rat paw induced by submaximal doses of carageenan (1 mg) and kaolin (10 mg). Potentiation of both oedemas by prostaglandin E_1 and arachidonic acid was maximal at 0.5 and 1.5 h respectively. Prostaglandin E_1, but not arachidonic acid, potentiated oedema induced by bradykinin (4.5 μg) and trypsin (50 μg). Oedema induced by 5-HT (1 μg), histamine (10 μg), dextran B (6 μg) and compound 48/80 was not potentiated by either prostaglandin E_1 or arachidonic acid. At

Table 2. Effects of prostaglandin E_1 (PGE_1, 0.1 μg) upon paw oedema induced by various phlogogenic agents

Phlogogenic agent	*(a) Oedema induced by agent (ml)*	*(b) Oedema induced by PGE_1 (ml)*	*(c) Oedema induced by combination (ml)*	*Difference c − (a + b) (ml)*
Carrageenan (1 mg)	0.29 ± 0.05	0.15 ± 0.03	0.86 ± 0.05	0.42 ± 0.07**
Kaolin (10 mg)	0.09 ± 0.03	0.16 ± 0.03	0.45 ± 0.04	0.20 ± 0.06*
Bradykinin (4.5 μg)	0.13 ± 0.03	0.08 ± 0.01	0.42 ± 0.05	0.21 ± 0.06*
Trypsin (50 μg)	0.56 ± 0.07	0.17 ± 0.03	1.02 ± 0.06	0.29 ± 0.09*
5-HT (1 μg)	0.40 ± 0.04	0.10 ± 0.02	0.52 ± 0.03	0.02 ± 0.05
Histamine (10 μg)	0.02 ± 0.02	0.15 ± 0.02	0.20 ± 0.03	0.03 ± 0.04
Dextran B (6 μg)	0.17 ± 0.04	0.11 ± 0.03	0.30 ± 0.04	0.02 ± 0.06
Compound 48/80 (1 μg)	0.30 ± 0.02	0.19 ± 0.03	0.59 ± 0.05	0.10 ± 0.06

All measurements were made 0.5 h after induction of oedema. Each value is the mean ± s.e. mean for a group of 7-10 rats. Where potentiation occurs significance is indicated by ** ($P < 0.001$) or * ($P < 0.01$)

0.5 h the inflammatory response to bradykinin (4.5 μg; 0.11 ± 0.04 ml) was also potentiated ($P < 0.05$) by prostaglandin E_2 (0.1 μg; 0.31 ± 0.03 ml), the oedema induced by prostaglandin E_2 alone at this time being 0.05 ± 0.04 ml.

Discussion

Prostaglandins E_1 and E_2 injected into the rat hindpaw induced only a slight to moderate oedema of brief duration, confirming findings by Arora *et al.* (1970), Glenn *et al.* (1972), Moncada *et al.* (1973) and Thomas & West (1974). Prostaglandin $F_{2\alpha}$ (250 ng) did not induce paw oedema and although such an action has been reported by Thomas & West (1974), μg quantities were required.

When either prostaglandin E_1 or E_2 is administered in combination with carrageenan, a significant potentiation of carrageenan-induced oedema results 0.5-1 h later. Prostaglandin $F_{2\alpha}$ is without effect upon carrageenan-induced oedema. A similar potentiation of carrageenan-induced oedema by prostaglandins of the E series has recently been demonstrated in the rat (Moncada *et al.*, 1973). However, Glenn *et al.* (1972) failed to demonstrate potentiation of carrageenan oedema by prostaglandin E_1 in the same test system.

The two unsaturated fatty acids, arachidonic acid and linolenic acid, exhibit no phlogogenic activity. However, arachidonic acid potentiated the response to carrageenan, as recently reported by Smith, Ford-Hutchinson, Elliot & Bolam (1974). Since arachidonic acid is a substrate for prostaglandin synthetase (Bergstrom, Danielsson & Samuelsson, 1964), whilst linolenic acid is not, it is possible that the potentiation by exogenous arachidonic acid is due to its conversion to prostaglandins. The susceptibility of prostaglandin synthetase to drugs varies in different tissues. For example, paracetamol inhibits effectively prostaglandin synthetase from rat brain (Flower & Vane, 1972) and rat platelets (Vargaftig & Dao Hai, 1973) but only weakly that from dog spleen (Flower *et al.*, 1972). Furthermore, whereas non-steroidal anti-inflammatory drugs inhibit the enzyme(s) extracted from dog spleen and sheep seminal vesicles (Flower *et al.*, 1972, Ham *et al.*, 1972), steroidal anti-inflammatory drugs like hydrocortisone have been shown to inhibit prostaglandin synthetase from crude homogenates of human skin (Greaves & McDonald-Gibson, 1972) but not that obtained from a microsomal fraction of rat skin (Greaves, Kingston & Pretty, 1975). The observation that only drugs whose anti-inflammatory mechanism of action may be attributed to prostaglandin synthetase inhibition (Vane, 1972) reduce arachidonic acid potentiation of carrageenan-induced oedema, strongly argues that this potentiation is due to conversion of arachidonic acid to prostaglandins. The prostaglandin synthetase in the oedematous rat paw appears to resemble more closely, in its susceptibility to drugs, the enzyme(s) from dog spleen and sheep seminal vesicles than prostaglandin synthetase from rat brain or human skin.

Infiltrating white blood cells constitute a possible source of prostaglandin synthetase in the oedematous paw. The migration of polymorphonuclear leucocytes and monocytes into the inflamed site is a feature of both carrageenan-induced rat paw oedema (Di Rosa & Willoughby, 1971) and kaolin-induced pleurisy in the rat (Vinegar, Truax & Selph, 1972). Phagocytosing rabbit polymorphonuclear leucocytes produce considerable quantities of prostaglandins (Higgs & Youlten, 1972) and are

Table 3. Effects of arachidonic acid (AA, 100 μg) upon rat paw oedema induced by various phlogogenic agents

Phlogogenic agent	*(a) Oedema induced by agent (ml)*	*(b) Oedema induced by AA (ml)*	*(c) Oedema induced by combination (ml)*	*Difference c − (a + b) (ml)*
Carrageenan (1 mg)	0.29 ± 0.06	0.02 ± 0.02	0.69 ± 0.05	0.38 ± 0.08**
Kaolin (10 mg)	0.14 ± 0.03	0.22 ± 0.02	0.59 ± 0.04	0.23 ± 0.05**
Bradykinin (4.5 μg)	0.09 ± 0.03	0.08 ± 0.02	0.13 ± 0.03	−0.04 ± 0.05
Trypsin (50 μg)	0.26 ± 0.03	0.02 ± 0.02	0.28 ± 0.03	0.00 ± 0.05
5-HT (1 μg)	0.22 ± 0.04	0.12 ± 0.01	0.37 ± 0.03	0.04 ± 0.05
Histamine (10 μg)	0.10 ± 0.04	0.04 ± 0.03	0.15 ± 0.02	0.01 ± 0.05
Dextran B (6 μg)	0.15 ± 0.03	−0.01 ± 0.01	0.17 ± 0.03	0.03 ± 0.03
Compound 48/80 (1 μg)	0.17 ± 0.04	0.15 ± 0.03	0.32 ± 0.03	0.00 ± 0.06

Legend as for Table 2 except that all measurements were made 1.5 h after induction of paw oedema.

capable of converting exogenous arachidonic acid to prostaglandins *in vitro* (McCall & Youlten, 1973). It is of interest to note that at the time of maximum arachidonic acid potentiation of carrageenan-induced oedema, i.e. at 1.5 h, a significant increase in polymorphonuclear leucocytes has been observed in the carrageenan-induced oedemous rat paw (Di Rosa & Willoughby, 1971).

5-HT, histamine and kinins have been shown to be involved in the mediation of carrageenan-induced paw oedema (Crunkhorn & Meacock, 1971). In addition, kinins have also been implicated in kaolin-induced oedema (Bonta & De Vos, 1967, Di Rosa, 1972). Neither prostaglandin E_1 nor arachidonic acid potentiated the inflammatory response to histamine or 5-HT or to agents releasing them such as dextran B or compound 48/80 (Jori, Bentivoglio & Garattini, 1961). On the other hand, bradykinin-induced paw oedema was potentiated by both prostaglandin E_1 and E_2 but not by arachidonic acid. Since bradykinin is not chemotactic for leucocytes (Ward, 1971) the inability of arachidonic acid to potentiate the response to bradykinin possibly results from the lack of conversion of fatty acid to prostaglandins due to the absence of these cells. The potentiation by prostaglandin E_2 (0.1 μg) of the bradykinin response does not agree with the observations of Thomas & West (1974), who used only 0.025 μg prostaglandin E_2. These authors suggested that their conditions may not have been optimal for demonstrating synergism between prostaglandin E_2 and bradykinin.

Prostaglandins potentiate other responses to kinins. Increases in vascular permeability in guinea-pig skin induced by intradermal injection of bradykinin may be potentiated by the concomitant administration of prostaglandin E_1 and E_2 and to a lesser extent by $F_{2\alpha}$ (Williams & Morley, 1973). In the rat skin, the response to bradykinin is potentiated by prostaglandin E_1 but not by low doses of E_2 (Thomas & West, 1974) whilst $F_{2\alpha}$ inhibits the response. In humans, prostaglandin E_1 potentiates the pain producing effect of intradermal injections of bradykinin (Ferreira, 1972). Similarly the nociceptive activity of bradykinin in the dog knee joint (Ferreira, Moncada & Vane, 1974) is potentiated by infusion of prostaglandins E_1 and E_2 but not by $F_{2\alpha}$. The observations of this study that oedema of the rat paw induced by both carrageenan and kaolin is potentiated by prostaglandins of the E series and by arachidonic acid may constitute yet another example of prostaglandin potentiation of a kinin response.

We thank M. Eccles for excellent technical assistance, Geigy Pharmaceuticals for pehnylbutazone; I.C.I. Ltd for fluocinoline acetonide; Sandoz A.G. for bradykinin and Syntex Pharmaceuticals for naproxen.

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(Received Febuary 14, 1975.
Revised May 2, 1975.)

Br. J. Pharmac (1975), 55, 57-64

MEDIATORS OF PASSIVE LUNG ANAPHYLAXIS IN THE RAT

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1 Passive lung anaphylaxis (PLA) was investigated in rats sensitized by the intravenous injection of high titre reaginic antiserum prepared in rats.

2 The effect of various pharmacological antagonists on anaphylactic bronchoconstriction *in vivo* were examined. An antihistamine (mepyramine), a kallikrein inactivator (aprotinin) or a prostaglandin synthesis inhibitor (aspirin) did not inhibit PLA, whereas an anti-5-hydroxytryptamine agent (methysergide) and an anti-slow reacting substance-A agent (FPL 55712) significantly reduced the response.

3 Isolated perfused lungs taken from sensitized rats released, on challenge with the sensitizing antigen, histamine, 5-hydroxytryptamine, slow reacting substance of anaphylaxis (SRS-A) and prostaglandins, but no rabbit aorta contracting substance (RCS).

4 Disodium cromoglycate inhibited both anaphylactic bronchoconstriction *in vivo* and the anaphylactic release of mediators *in vitro*. Inhibition *in vivo* was dose-related.

5 Mediators from the intestine, the primary shock organ of anaphylaxis in the rat, did not contribute to the lung response.

6 Vagal reflex pathways were found not to be important in PLA *in vivo*.

7 The relationship between the mediators released following antigen challenge of passively sensitized rat lung *in vitro* and passive lung anaphylaxis *in vivo* is discussed.

Introduction

One of the tests which has been widely used in the search for new compounds effective in the treatment of bronchial asthma, is passive cutaneous anaphylaxis (PCA) in the rat induced by homologous reagin (IgE)-like antibodies (Goose & Blair, 1969). These antibodies have properties (e.g. they are heat labile, non-precipitating and act with long latency) similar to human reagins which are associated with immediate hypersensitivity in man. No study has been reported of anaphylactic bronchoconstriction in rats passively sensitized with rat IgE. A study of this reaction with regard to mediator release and activity of the anti-asthma agent, disodium cromoglycate, both *in vitro* and *in vivo* has therefore been made. A preliminary report of this work was communicated to a Joint Meeting of the British Pharmacological Society and the Deutsche Pharmakologische Gessellschaft (Farmer, Richards, Sheard & Woods, 1973).

Methods

Passive sensitization

Female Sprague Dawley rats weighing 200-250 g were sensitized by the injection of conalbumin 5 mg/kg intramuscularly and 0.5 ml *Bordetella pertussis* vaccine 2×10^{10} organisms intraperitoneally, on day 0. On day 10 the animals were injected with 4×10^3 larvae *Nippostrongylus brasiliensis* subcutaneously to potentiate the production of antibodies to conalbumin (Orr & Blair, 1969). On day 24 or 25, the blood was collected by cardiac puncture under anaesthesia and the antisera collected, pooled and stored at -20°C. Groups of 100 or 200 rats were used to provide a large bulk of antiserum. The titre (or highest effective dilution which gave a mean 72 h PCA response of 5 mm in diameter in a rat) of the antiserum used was 1:512. Wistar rats were passively sensitized by intravenous injection of 0.5-1 ml of this high titre antiserum.

Passive lung anaphylaxis in vivo

Twenty-four hours after sensitization, rats were anaesthetized with intraperitoneal pentobarbitone sodium 80-100 mg/kg, a dose sufficient to abolish spontaneous respiration. The tail vein of each rat was cannulated with a hypodermic needle cut from its mount and inserted in narrow polythene tubing to provide a flexible connection to a syringe for intravenous injection. Airways resistance was then measured by the overflow technique of Konzett & Rossler (1940) as modified by Burden, Parkes & Gardiner (1971). The trachea was cannulated and the animal ventilated by means of a Starling miniature respiration pump at a rate of 72 strokes/minute. Inflation pressure was kept constant at 9 cm water by means of a water valve. The air overflow from the water valve passed through a pneumotachograph tube connected to a differential air pressure transducer. Changes in overflow were displayed on a Devices recorder. The animals were challenged with egg albumin 25 mg/kg. Anaphylactic bronchoconstriction was recorded as % of the maximal overflow (obtained by clamping off the trachea). The inhibitory effect of antagonists was given by the formula:

$$\frac{\%\ \text{bronchoconstriction in control group} - \%\ \text{bronchoconstriction in test group}}{\%\ \text{bronchoconstriction in control group}} \times 100.$$

Evisceration of rats

The abdomen was opened, the viscera exposed and 3 branches of the superior mesenteric artery (ileo-colic, colic and pancreatic-duodenal) tied off. The intestines were then removed from just below the stomach to the anus. In 'sham' operations, the intestines and blood vessels were left intact.

Passive lung anaphylaxis in vitro

Rats were killed 24 h after sensitization and the lungs removed and perfused via the pulmonary artery with Krebs solution gassed with 95% O_2 and 5% CO_2, as described by Piper & Vane (1969) for guinea-pig lungs. The Krebs solution had the following composition (mM): NaCl 118, $MgSO_4 7H_2O$ 1.17, $NaHCO_3$ 25.0, glucose 5.6, KCl 4.7, $CaCl_2 6H_2O$ 2.5, KH_2PO_4 1.2. The effluent was superfused over six tissues at 6 ml/min using a Watson-Marlow flow inducer. The tissues were suspended in polycarbonate chambers and arranged in two banks of three. The actions of acetylcholine, histamine, 5-hydroxytryptamine (5-HT) and catecholamines were eliminated, except where indicated, with a combination of antagonists (referred to as combined antagonists), consisting of atropine sulphate 0.1 μg/ml, mepyramine maleate 0.1 μg/ml, methysergide bimaleate 0.1 μg/ml, phenoxybenzamine hydrochloride 0.1 μg/ml and propranolol hydrochloride 2 μg/ml. These antagonists were infused at 0.1 ml/min into the superfusing fluid so that the antagonists reached some or all of the assay tissues.

Histamine was assayed on the cat terminal ileum treated with the combined antagonists excluding mepyramine and methysergide.

Several tissues were tested for sensitivity to 5-HT. In preliminary experiments rat stomach strips were used, but it proved difficult to separate the actions of 5-HT and prostaglandins. The most useful tissue for 5-HT assay was the rabbit aorta. After it had been verified that no rabbit aorta contracting substance (RCS) was released from sensitized rat lungs by antigen challenge, as found by Piper & Walker (1973) in actively sensitized rats, the rabbit aorta was used regularly for the detection of 5-HT. Higher sensitivity was found in spirally cut strips taken from young female rabbits. The superfusion fluid for this tissue contained the combined antagonists excluding methysergide.

A guinea-pig ileum treated with the combined antagonists was used to detect slow-reacting substance (SRS)-A. Standard responses were obtained with crude SRS-A prepared from guinea-pig lungs.

Prostaglandins were assayed on the rat stomach strip, chick rectum and rat colon treated with the combined antagonists (Piper & Vane, 1969).

Standard solutions of agonists were injected either in known volumes (1 ml or less as a bolus injection) before the roller pump to avoid any injection artefacts, or by slow infusion (0.1 ml/min) for 1 or 3 min over the assay tissues. Bolus injection was the most useful procedure when a series of bench samples were to be assayed, while slow infusion produced tissue responses which more closely resembled those obtained after challenge of sensitized lungs suspended above the assay tissues. The isolated lungs were challenged with 400 μg of 5 x crystallized egg albumin solution.

Drugs and reagents

The drugs and other agents used were histamine acid phosphate (BDH), 5-hydroxytryptamine sulphate (BDH), bradykinin (Sandoz), prostaglandin $F_{2\alpha}$ (Upjohn), prostaglandin E_2 (Cambrian Chemicals), methysergide bimaleate (Sandoz), mepyramine maleate (May & Baker), aprotinin (Trasylol, Bayer), disodium cromoglycate (Fisons), FPL 55712 (Fisons), atropine sulphate (Sigma), phenoxybenzamine hydrochloride (ICI), propranolol hydrochloride (ICI), sodium pentobarbitone (May & Baker), acetylsalicylic acid (BDH), egg albumin (BDH), conalbumin Type II (Sigma), 5 x crystallized egg albumin (Koch-Light) and *B. pertussis* vaccine (Burroughs Wellcome).

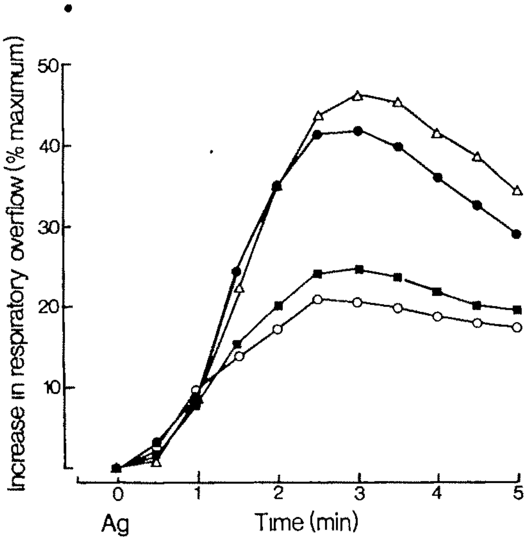

Figure 1 Time course of anaphylactic bronchoconstriction in passively sensitized rats. (●) Control response; (△) response in animals pretreated with mepyramine (10 mg/kg, i.p. 30 min before antigen), (■) methysergide (4 mg/kg, i.v. 15 min before antigen) or (○) FPL 55712 (10 mg/kg, i.v. immediately before antigen). ($n = 9$)

Results

Effect of heat treatment of reaginic antiserum on its ability to sensitize rats for anaphylactic bronchoconstriction in vivo

One group of 7 rats was sensitized with unheated serum and another with serum which had been heated at 56°C for 1 hour. The increase in respiratory overflow (% maximum mean ± s.e. mean) was, in the first group 58 ± 8.3 and in the second group 10.7 ± 1.2 ($n = 7$). These results suggested that a heat labile reagin-like antibody was responsible for the greater part of the lung anaphylactic responses produced in these experiments.

Effect of pharmacological antagonists on anaphylactic bronchoconstriction in vivo

Figure 1 shows the time course of anaphylactic bronchoconstriction in untreated rats and in rats after pretreatment with an antihistamine (mepyramine 10 mg/kg i.p. 30 min before antigen), an anti-5-HT agent (methysergide, 4 mg/kg i.v. 15 min before antigen) or a selective antagonist of SRS-A (FPL 55712 10 mg/kg i.v. immediately before antigen; Augstein, Farmer, Lee, Sheard & Tattersall, 1973). The increase in respiratory overflow (% maximum) was as follows: control group 42.7 ± 7.2, mepyramine-treated group 47.4 ± 7.3, methysergide-treated group 25.0 ± 1.7 and FPL 55712-treated group 21.7 ± 4.4. The % inhibition produced by the drugs was nil, 41.3 ($P < 0.05$) and 49.2 ($P < 0.25$), respectively.

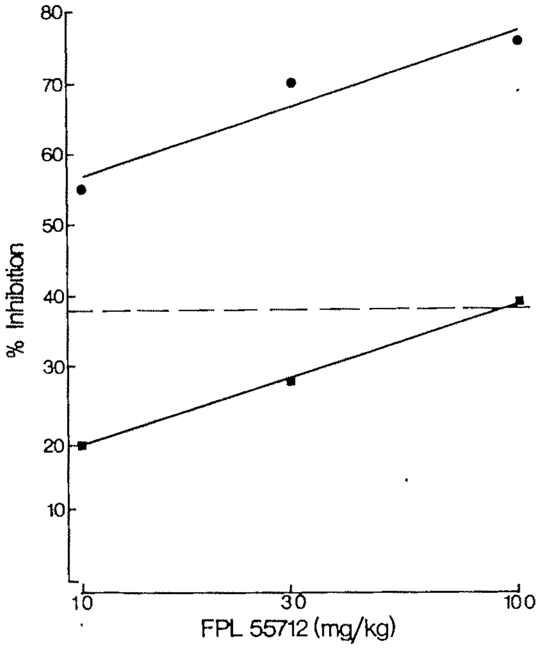

Figure 2 Inhibition of anaphylactic bronchoconstriction in passively sensitized rats after pretreatment with methysergide (4 mg/kg, i.v. 15 min before antigen) indicated by dashed line; (■) FPL 55712, and (●) FPL 55712 + methysergide (4 mg/kg) ($n = 7$).

The dose of FPL 55712 used in these experiments inhibits PCA in the rat (unpublished results). A further experiment was therefore performed to determine if the effects of FPL 55712 and methysergide were additive in PLA. FPL 55712, 1, 3 and 10 mg/kg was tested against anaphylactic bronchoconstriction in the presence and absence of methysergide 4 mg/kg. The results (Figure 2) show that the effects of the compounds were additive at each dose of FPL 55712.

FPL 55712 (10 mg/kg) did not inhibit bronchoconstrictor responses produced by intravenous injection of 5-HT (12.5 μg/kg) in the rat, which confirmed the selectivity found *in vitro* with FPL 55712 (Augstein *et al.*, 1973). Intravenous injection of other possible mediators of anaphylaxis (histamine, bradykinin, prostaglandins E_2 or $F_{2\alpha}$ at doses up to 100 μg/kg) did not produce bronchoconstriction in the rat, although all of these agents produced a fall in blood pressure.

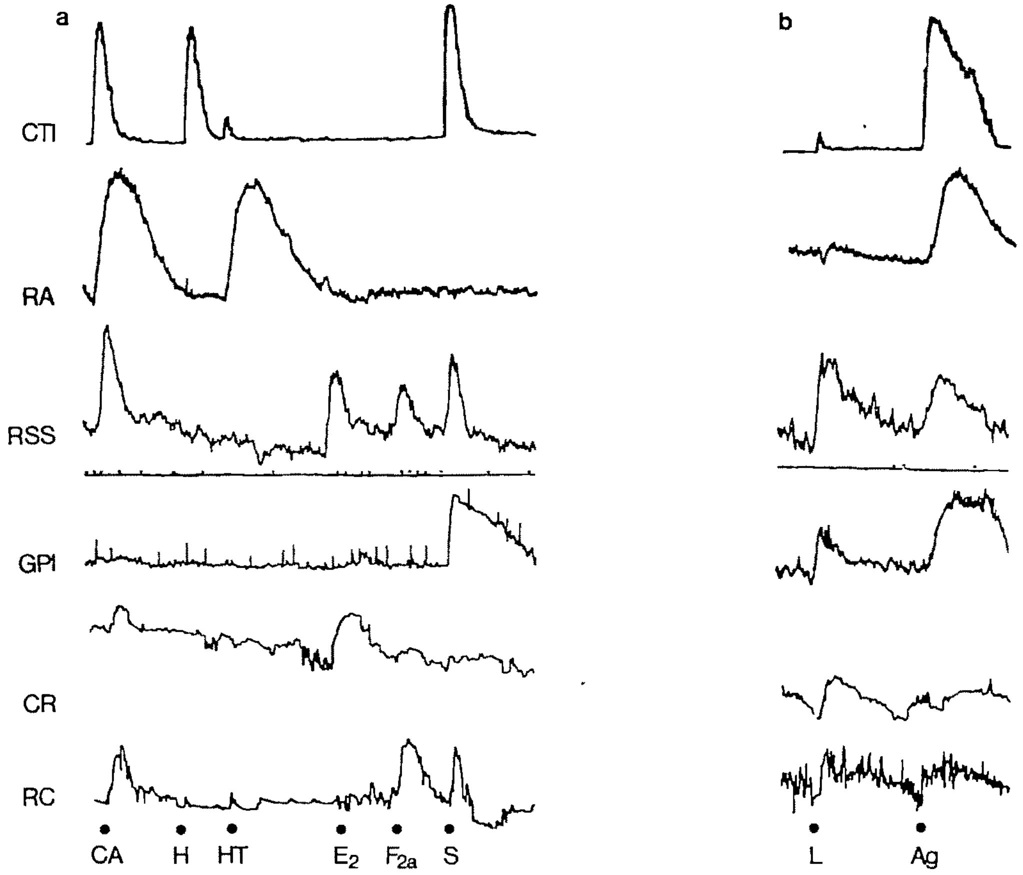

Figure 3 Mediators released after antigen challenge of lungs taken from passively sensitized rats: (a) standard solution of individual agonists, histamine 20 ng/ml (H), 5-hydroxytryptamine 20 ng/ml (HT), prostaglandins E_2 2 ng/ml and $F_{2\alpha}$ 2 ng/ml and crude SRS-A 1.0 ml (S); and combined agonists (CA) excluding SRS-A; superfused over cat terminal ileum (CTI), rabbit aorta (RA), rat stomach strip (RSS), guinea-pig ileum (GPI), chick rectum (CR) and rat colon (RC); (b) the effluent from isolated lungs superfused over the same assay tissues, L = lungs suspended above the assay tissues, Ag = injection of 400 µg of 5 x crystallized egg albumin. Selective antagonists were superfused over the assay tissues as described in the text.

Aspirin pretreatment in vivo

Following treatment with aspirin (5 mg/kg i.v.) 10 min before challenge, the % maximum bronchoconstriction (mean ± s.e. mean) was 86.0 ± 2.9 (n = 5) compared to 67.1 ± 16.2 (n = 5) for controls. The difference between these responses was not significant ($P > 0.05$).

Aprotinin pretreatment in vivo

Intraperitoneal injection of aprotinin, 100,000 kallikrein-inhibitor units/kg 10 min before challenge, produced a slight inhibition of the bronchoconstrictor response, but this was not significant ($P > 0.05$). The % maximum broncho constriction (mean ± s.e. mean) was 55.0 ± 11.5 (n = 5) compared to 63.6 ± 7.0 (n = 5) for control rats.

Influence of mediators released from the intestine in vivo

There was no significant difference ($P > 0.05$) in the anaphylactic bronchoconstriction obtained in eviscerated or 'sham' operated animals. The % maximum bronchoconstriction (mean ± s.e. mean) was 44.9 ± 10.2 (n = 7) in eviscerated rats and 42.8 ± 7.1 (n = 7) in 'sham' eviscerated rats.

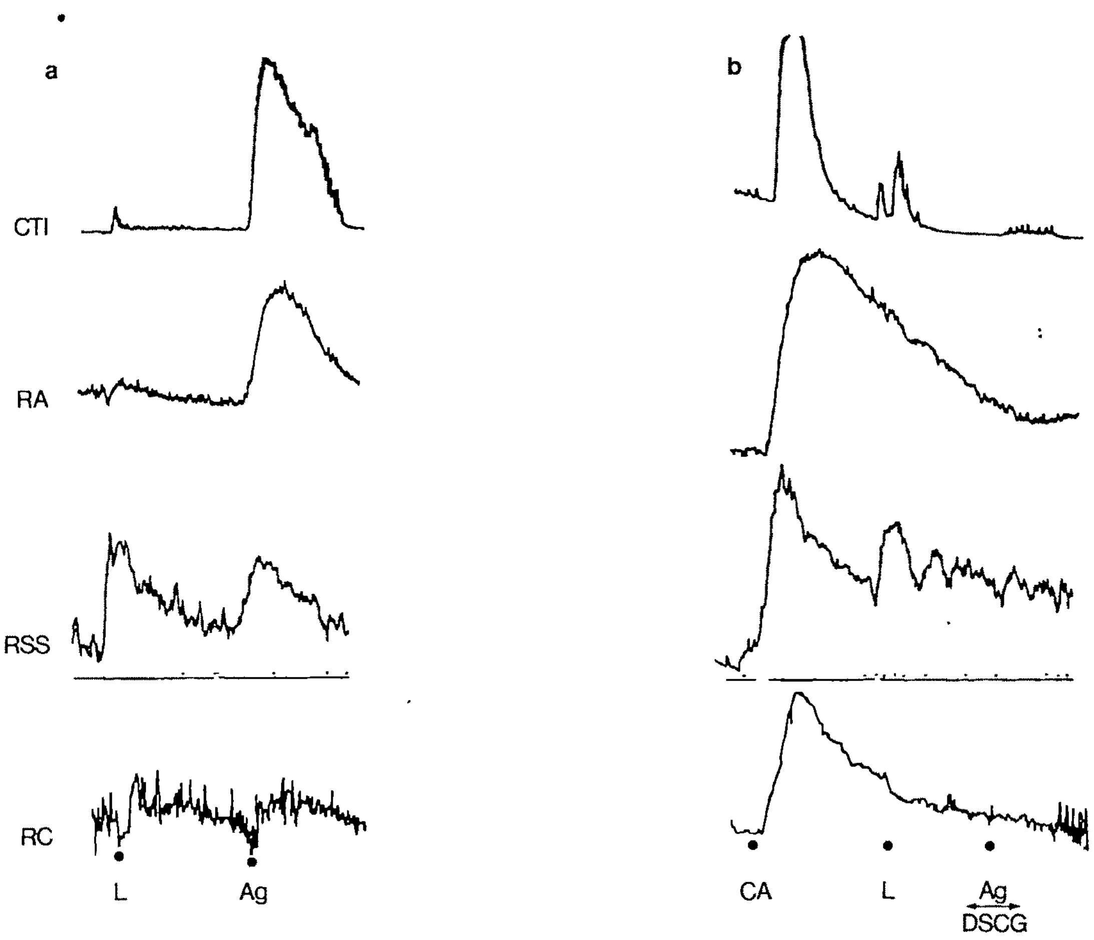

Figure 4 Inhibition by disodium cromoglycate (DSCG) of release of mediators after antigen challenge of lungs taken from passively sensitized rats; (a) control lungs; (b) lungs perfused with DSCG 10 μg/ml for 1 min before and 5 min after antigen challenge. Other details as in Figure 3.

Vagal involvement in vivo

In groups of 6 rats there was no significant difference ($P > 0.05$) in the anaphylactic bronchoconstriction obtained in bilaterally vagosympathectomized animals (64.5 ± 6.1) or in 'sham' operated animals (71.0 ± 10.8).

Mediators released from passively sensitized lung in vitro

After challenge of isolated perfused lungs taken from passively sensitized rats, the perfusing fluid was allowed to superfuse a series of assay tissues (Figure 3). The release of histamine was demonstrated by a contraction of the cat terminal ileum; of 5-HT by a methysergide-sensitive contraction of the rabbit aorta; of SRS-A by a slow, prolonged contraction of the guinea-pig ileum superfused with combined antagonists; and of prostaglandins by contractions of the rat stomach strip, chick rectum and rat colon. It was noticeable that prostaglandins could only be detected after severe shock when large amounts of the other mediators were also released. In some experiments only histamine and 5-HT and no prostaglandins were released. After a second antigen challenge no mediators could be detected in the perfusing fluid.

No contraction of a rabbit aortic strip superfused with a combination of antagonists including methysergide was seen after challenge of 8 sets of sensitized rat lungs. That lung shock had actually taken place was shown by the presence of the other mediators and the contraction of a second aortic strip which was not superfused with methysergide. These results agree with the findings of Piper & Walker (1973) that RCS is released after mechanical stimulation, but not after antigen challenge of lungs taken from actively sensitized rats.

Effects of disodium cromoglycate on PLA in vivo *and* in vitro

In vivo dose-dependent inhibition of passive lung anaphylaxis was obtained by disodium cromoglycate (DSCG) given intravenously 1 min before challenge in groups of 7 rats. Doses of 0.4, 1, 2 and 5 mg/kg DSCG gave respectively 28.2, 46.6, 52.3 and 84.5% inhibition of PLA, the ID_{50} being 1.25 mg/kg.

In vitro DSCG (10 or 100 μg/ml) was infused through the isolated lungs from 1 min before antigen challenge until 5 min after challenge. DSCG at 100 μg/ml totally inhibited mediator release in 5 out of 6 rats and at 10 μg/ml in 3 out of 7 rats (Figure 4). Sixteen control sets of lungs all showed some mediator release.

Discussion

A chemical substance can be accepted as a mediator of a biological reaction only if several criteria are satisfied (Vane, 1972a). The present experiments allow some conclusions about the contribution of various potential mediators to anaphylactic bronchoconstriction in rats passively sensitized with reaginic antiserum.

The evidence that 5-HT participates as a mediator of PLA in the rat is conclusive. 5-HT injection into the normal rat produced bronchoconstriction. Methysergide, an antagonist of 5-HT, reduced anaphylactic bronchoconstriction *in vivo*. Rat lungs contain relatively high quantities of 5-HT (Weissbach, Waalkes & Udenfriend, 1958, 3.5 μg/g; Sadavongvivad, 1970, 1.91 μg/g) and we have shown that 5-HT can be released from rat lungs during PLA *in vitro*.

Although histamine is present in rat lungs (Aviado & Sadavongvivad, 1970, 5.0 μg/g) and can be released during anaphylactic shock *in vitro*, histamine injection did not produce bronchoconstriction *in vivo*, nor did mepyramine reduce anaphylactic bronchoconstriction *in vivo*. It appears therefore that histamine is not an important mediator of anaphylactic bronchoconstriction in the rat, in contrast to conditions in the guinea-pig (Parrat & West, 1957; Austen, 1963).

The lungs of actively sensitized guinea-pigs release SRS-A upon challenge with specific antigen (Brocklehurst, 1960). We have demonstrated the release of SRS-A following antigen challenge of lungs from passively sensitized rats *in vitro*. In addition, the partial inhibition of anaphylactic bronchoconstriction in passively sensitized rats *in vivo* by FPL 55712 suggests that SRS-A is involved in this response. FPL 55712 has some anti-PCA activity in the rat, which might suggest that the inhibitory effect on anaphylactic bronchoconstriction which we obtained was due to a general anti-allergic property of this compound. However, the additive effects of FPL 55712 and methysergide in the present experiments suggest that FPL 55712 acts mainly as an antagonist of SRS-A. Conclusive evidence for SRS-A involvement in rat PLA will be possible only when rat SRS-A has been purified.

Main (1964) and Horton (1969) have suggested that prostaglandins are normally involved in the local control of bronchial smooth muscle tone. This view is supported by Vane (1972b) who suggests that prostaglandins function as homeostatic regulators in the lung by modulating the effects of other mediators rather than by producing anaphylactic bronchoconstriction themselves. Our results to some extent support this view. Aspirin pretreatment did not modify PLA *in vivo*. During *in vitro* experiments prostaglandins were detected in the perfusates from lungs only after severe anaphylactic shock. Moreover, the ratio of prostaglandins to other mediators released was far higher during the mechanical stimulation involved in setting up the lungs than during anaphylactic shock (Figure 3). During anaphylactic shock in rats bradykinin is found in the blood (Csaba & Went, 1971), bradykininogen levels fall and kinin-forming activity is increased (Dawson, Starr & West, 1966; Csaba & Went, 1971). Because no true bradykinin antagonists were available, aprotinin which inhibits kinin formation was used. Aprotinin did not modify anaphylactic bronchoconstriction. In addition, the injection of bradykinin into rats did not induce bronchoconstriction. This argues against bradykinin being a mediator of anaphylactic bronchoconstriction in the rat.

In the experiments reported here, DSCG produced a dose-related inhibition of PLA *in vivo* and complete inhibition of mediator release *in vitro* (Figure 4). This is in contrast to the 'bell-shaped' dose-inhibitory response curve produced against anaphylactic bronchoconstriction in actively sensitized rats (Church, Collier & James, 1972).

DSCG is thought to act primarily at the cellular level by preventing the release of tissue damaging substances following antigen interaction with mast cells sensitized with IgE (Cox, 1971).

Evidence has been presented that DSCG blocks cyclic adenosine 3′,5′-monophosphate (AMP) phosphodiesterase *in vitro* (Roy & Warren, 1974). However, the drug has no spasmolytic activity on smooth muscle and therefore, probably has no effect on smooth muscle phosphodiesterase. Taylor, Francis, Sheldon & Roitt (1974), carried out experiments using rat PCA and peritoneal mast cell degranulation which led them to suggest that the anti-asthmatic activity of DSCG might be due to its inhibitory effect on a specific mast cell phosphodiesterase iso-enzyme. However, experiments of Koopman, Orange & Austen (1970) had previously suggested that the action of DSCG in inhibiting antigen-induced release of SRS-A from the rat IgE-sensitized peritoneal cavity of rats was independent of the cyclic AMP system. Thus, the relevance of phosphodiesterase inhibition by DSCG to its anti-asthmatic effect, particularly when considered in relation to the respective doses needed to exert these effects, is still uncertain.

The unusual bell-shaped dose-response curve with DSCG in actively sensitized rats *in vivo* can probably best be explained by the presence of antibodies other than IgE, which may contribute to, or initiate events leading to, anaphylactic bronchoconstriction in these animals. In contrast, in the passively sensitized rat, the long latency and heat lability of the antibodies involved in the anaphylactic bronchoconstriction suggest that the reaction is primarily due to interaction of antigen with IgE attached to the lung tissue. Thirteen other compounds (each of which produced a dose-related inhibition of rat PLA) had almost identical activity, relative to DSCG, both in rat PLA and PCA and therefore, PLA was no more predictive as a screen for anti-allergic activity than PCA (Farmer *et al.*, 1973).

Orange, Stechschulte & Austen (1970), have demonstrated the release of histamine and/or SRS-A during passive peritoneal anaphylaxis in the rat. Mediators released were dependent on the immunoglobulins involved. As our experiments with eviscerated animals showed, mediators released from the intestine, considered to be the primary 'shock' organ of anaphylaxis in the rat (Sanyal & West, 1958; West, 1959), did not contribute to anaphylactic bronchoconstriction. The mediators which produce bronchoconstriction *in vivo* are probably released from the lungs themselves.

It has been suggested that vagal reflexes are partly responsible for anaphylactic bronchoconstriction in guinea-pigs (Mills & Widdicombe, 1970), dogs (Gold, Kessler & Yu, 1972) and rabbits (Karczewski & Widdicombe, 1969). In our experiments, vagotomy did not modify PLA in the rat. This species difference could be significant in the search for a predictive animal test for anti-allergic activity in man. However, the involvement of vagal reflexes in human bronchial asthma has not been clearly established. Some workers have shown atropine to be an effective inhibitor of antigen-induced bronchoconstriction in asthmatic patients (Yu, Galant & Gold, 1972) while others report little (Itkin & Anand, 1970), or no effect (Orie, Van Lookeren Campagne, Knol, Booij-Noord & De Vries, 1973, Rosenthal, Summer, Permutt & Norman, 1974). The relevance of anaphylactic bronchoconstriction in rats (or other animals) to asthma in man, with respect to vagal reflex involvement, cannot be resolved at present.

We are grateful to Mrs Diana R. Allebon and Miss Mary E. Leeson for technical assistance, and to Dr Priscilla J. Piper and Mr T.W. Smith for advice on the technique of cascade superfusion.

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(Received February 24, 1975.
Revised May 31, 1975.)

Br. J. Pharmac. (1975), 55, 65-68

ANTI-INFLAMMATORY ACTION OF GLUCAGON IN RATS

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1 Subcutaneous administration of glucagon (1 and 0.5 mg/kg) 30 min before the injection of carrageenin or dextran into the rat's paw reduced oedema and the local exudation of Evans blue previously given intravenously.

2 The effect persisted after removal of the adrenal medulla but not after adrenalectomy.

3 When glucagon (1 mg/kg, s.c.) was given daily after a local reaction to Freund's adjuvant injected into the paw had developed, a decrease in the reaction was observed up to 12 days. Blood sugar levels remained within the normal range.

4 Glucagon may exert an anti-inflammatory effect through the release of adrenal corticosteroids and thus help modulate inflammatory reactions.

Introduction

An interplay of hormones seems to influence the development of inflammatory reactions (Garcia Leme & Schapoval, 1975). Insulin may act as a pro-inflammatory agent (Garcia Leme, Hamamura, Migliorini & Leite, 1973b; Garcia Leme, Böhm, Migliorini & Souza, 1974). Most of the actions of glucagon oppose those of insulin. The glucagon-producing alpha cells and the insulin-producing beta cells of pancreatic islets are regarded as a single functional unit performing opposing functions (Unger, 1970).

The present experiments were therefore undertaken to investigate whether glucagon affects inflammatory reactions.

Methods

Male Sprague Dawley (Holtzman) rats weighing between 200-250 g were used.

Production of oedema in rat paws

Carrageenin (0.5 mg/ml) or dextran (1.0 mg/ml) was dissolved in distilled water and 0.1 ml injected into the subplantar area of one of the hind paws; the other was injected with 0.1 ml of 0.9% w/v NaCl solution (saline). The volumes of the paws up to the tibio-tarsal articulation were determined by plethysmography at various time intervals with a modification of the apparatus described by Winder, Wax & Been (1957). Results are expressed as percentage increase in relation to the initial volume of the paw. Control values (saline-injected paws) were subtracted from values obtained in carrageenin or dextran-injected paws. In these groups the animals were anaesthetized with pentobarbitone sodium (20-40 mg/kg, i.p.). Complete Freund's adjuvant was used as commercially available; 0.1 ml of the suspension was injected into the subplantar area of one of the hind paws of unanaesthetized rats. The volumes of the paws were determined every three days. The percentage increase in relation to the initial volume, at 3 days after the injection of adjuvant, was designated as 100. This was done to minimize individual variations and because drugs were applied from the 3rd to 15th day after adjuvant injections.

Double coaxial perfusion of the rat paws

The subcutaneous space of both hind paws was perfused (Garcia Leme, Hamamura & Rocha e Silva, 1970) through polyethylene tubing (3 mm external and 2 mm internal diameter) introduced into the subcutaneous space of the distal part of the paws through a small incision high in the limbs. The perfusing saline solution (37° C) reached the subcutaneous space through a narrower inner tube and was collected through the outer one, the flow being adjusted to 1 ml/ 10 minutes. The perfusions were carried out for 30 minutes. Evans blue (40 mg/kg; 2% aqueous solution) was injected intravenously 5 min before the perfusions and the amount of dye present in the perfusates estimated by spectrophotometry at 600 nm. Control values (dye in perfusates from saline-injected paws) were subtracted from test values (dye in perfusates from carrageenin or dextran-injected paws).

Removal of adrenal glands of medullae

The adrenal glands were removed under ether anaesthesia, by the dorsal approach. After adrenalectomy, rats were supplied with saline and water *ad lib.*, and were tested 72-80 h later. No functioning accessory adrenocortical tissue develops within 72-80 hours. Sham-operated animals were also tested at 72-80 h post-operatively.

The medulla was expressed through a small incision in the capsule. Operated and sham-operated rats were tested 72-80 h post-operatively.

Blood sugar levels

These were estimated by the method of King & Garner (1947) in 0.05 ml whole blood from a tail vein.

Drugs

The following drugs were used: dextran, mol. wt. 60,000-90,000 (Nutritional Biochemical Corp.), carrageenin, mol. wt. 60,000-100,000 (Merck, Sharp & Dohme), complete Freund's bacto-adjuvant (Difco Labs.), crystalline glucagon (Lilly), Evans blue (E. Merck AG.), pentobarbitone sodium (Nembutal, Abbott), and indomethacin (Merck, Sharp & Dohme).

Results

The subcutaneous administration of 0.5 or 1 mg/kg glucagon attenuated the development of oedema in paws injected with carrageenin or dextran 30 min later. Both doses of glucagon produced similar inhibition of the swelling (Figure 1a,b), possibly because they were absorbed from subcutaneous tissue at similar rates. Measurement of paw volumes made 0.5, 1, 2, 4, 6 and 24 h after the injection of the irritants showed in glucagon-treated rats significantly less oedema than the controls ($P < 0.01$, Student's t test), with the exception of those obtained at 24 hours. Blood sugar levels, determined 30 min after glucagon (0.5 or 1.0 mg) were 94-142 and 97-147 mg/100 ml blood respectively, and 74-130 mg/100 ml blood in the control group.

The exudation of Evans blue was also reduced in animals given 1 mg/kg glucagon, subcutaneously, 30 min before the injection of carrageenin or dextran (Figure 1c,d). Significant reduction ($P < 0.01$, Student's t test) was observed 0.5 and 1 h after the injection of the irritants. The leakage of dye was increased only during the 60 min following the injection of dextran. This confirmed findings with carrageenin (Garcia Leme, Hamamura, Leite & Rocha e Silva, 1973). Therefore the effects of drugs upon increased vascular permeability, as measured by the perfusion technique, should be evaluated during the one hour period following the injection of the irritants. Blood sugar levels were between 84-140 and 78-140 mg/100 ml blood in glucagon-treated and control animals, respectively.

Glucagon (1 mg/kg, s.c.) given to adrenalectomized rats 30 min prior to the injection of carrageenin or dextran did not reduce paw swelling (Figure 2a). No significant differences were found (Student's t test) between mean values in glucagon-treated and those in adrenalectomized or mock-adrenalectomized animals. However, glucagon (1 mg/kg, s.c.) decreased oedema produced in adrenal-demedullated animals by carrageenin or dextran for up to 6 or 4 h ($P < 0.01$) respectively (Figure 2b).

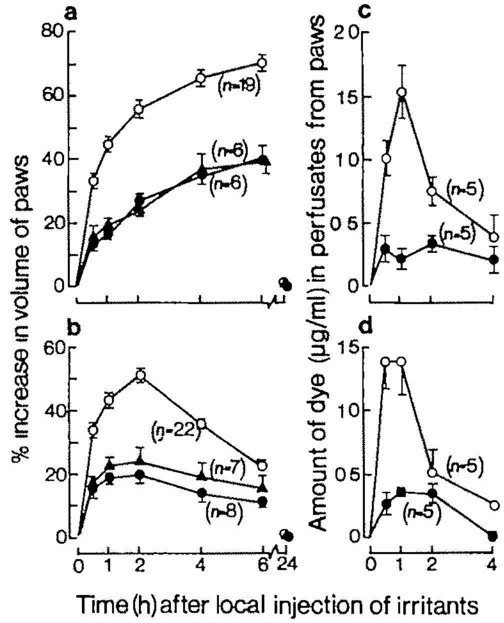

Figure 1 Oedema formation in rat paws after the local injection of (a) carrageenin (50 µg) or (b) dextran (100 µg) at 0 hours. (c) Dye (Evans blue, injected, i.v.) concentration in perfusates from rat paws with carrageenin or (d) dextran oedemas. (o) Controls; (▲) rats pretreated with glucagon (0.5 mg/kg), s.c.), 30 min before the injections of the irritants; (●) rats pretreated with glucagon (1.0 mg/kg, s.c.) 30 min before the injections of the irritants. Each point is the mean value; vertical bars show s.e. mean. n = number of animals used to obtain each point.

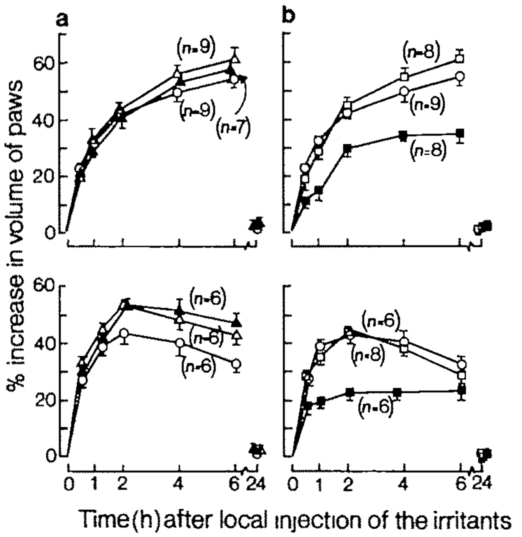

Figure 2 Oedema formation in rat paws injected with carrageenin (50 μg) (upper graphs) or dextran (100 μg) (lower graphs). (a) Results obtained in adrenalectomized (△), sham-adrenalectomized (○) or in adrenalectomized rats pretreated with glucagon (1 mg/kg, s.c.) 30 min before the injection of the irritants (▲); (b) Results obtained in adrenal-demedullated (□), sham-operated (○) or in adrenal-demedullated rats pretreated with glucagon (1 mg/kg, s.c.) 30 min before the injection of the irritants (■). Each point is the mean value; vertical bars indicate s.e. mean. n = number of animals used to obtain each point.

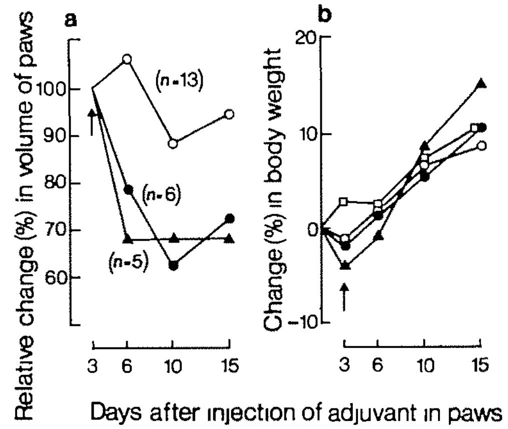

Figure 3 (a) Effect of daily injections of glucagon (1 mg/kg, s.c.) (●), or indomethacin (2 mg/kg, i.p.) (▲) from the 3rd (arrow) to the 15th day on oedema induced in the rat's paw by the local injection of 0.1 ml Freund's adjuvant. (○) Untreated rats injected with adjuvant. Percentage increase in relation to the initial volume of the paws, estimated 3 days after the injection of adjuvant, was arbitrarily taken as 100 and subsequent changes expressed in relation to it. (b) Changes in body weight during the observation period. (□) Six untreated rats injected with 0.1 ml saline in the paw; other symbols as in (a). n = number of animals used to obtain each point.

Daily administration of glucagon (1 mg/kg, s.c.) from the 3rd to 15th day after injection of Freund's adjuvant into the rat's paw, produced a decrease of the already developed oedema ($P < 0.01$, Student's t test). A similar reduction was produced by daily injections of indomethacin (2 mg/kg, i.p.) (Figure 3a). In the three days following Freund's adjuvant injection rats lost some weight as compared to a control group. However, after this period, treated and untreated animals gained as much weight as the control group (Figure 3b). Blood sugar levels determined in all groups at the beginning of the experiments and at days 6, 10 and 15 were within the normal range.

Discussion

Our results suggest that glucagon may have an anti-inflammatory action which is likely to depend on release of corticosteroids as it was ineffective in adrenalectomized rats.

Glucagon was effective when given before carrageenin or dextran or when given after the initial signs of adjuvant arthritis had developed. In the latter case daily doses of glucagon for 12 days were as effective as daily doses of indomethacin (2 mg/kg). However, indomethacin does not act through the release of corticosteroids (Garcia Leme & Schapoval, 1975). As pointed out by Whitehouse (1973/74), administration of a drug after the onset of inflammation bears more relationship to its potential use as an anti-inflammatory agent than prophylactic administration. The daily administration of glucagon may impair the regulation of blood sugar levels. However, daily doses of glucagon (1 mg/kg, s.c.) did not produce a persistent deviation from the normal range.

Serum concentrations of corticosterone were increased in rats after paw injections of carrageenin (Garcia Leme & Schapoval, 1975). Subcutaneous administration of corticosterone reduced the oedema resulting from a subsequent injection of carrageenin (Garcia Leme & Schapoval, 1975) and attenuated the vascular responses to histamine and 5-hydroxytryptamine given intradermally (Garcia Leme & Wilhelm, 1975). Plasma glucagon levels are elevated in patients with infections (Santeusanio, Rocha, Faloona, Muller &

Unger, 1972) and in animals following intravenous injection of bacterial pyrogens (Bloom, 1973). Release of glucagon was also induced by stress (Bloom, Daniel, Johnston, Ogawa & Pratt, 1973).

Catecholamine release from the adrenal glands does not seem to be required for the anti-inflammatory action of glucagon. However, in man, adrenaline stimulates glucagon secretion (Gerich, Karam & Forsham, 1973).

Significant glucagon release during sympathetic nerve stimulation has been seen in the cat (Esterhuizen & Howell, 1970), dog (Renold, 1972) and calf (Bloom, 1973; Bloom, Edwards & Vaughan, 1973). Bloom *et al.* (1973) deduced from their results that any stimulus of sufficient intensity to constitute a 'stress' may cause a non-specific increase in sympathetic activity, which could raise plasma glucagon concentrations.

Similar mechanisms may occur during inflammatory reactions. Catecholamines from the adrenal glands or increased sympathetic activity may enhance glucagon secretion which in turn would increase blood levels of corticosteroids and thus suppress inflammation.

Stimulation of the hypothalamo-pituitary-adrenal axis with release of corticosteroids was observed during the early phases of acute inflammatory reactions (Garcia Leme & Schapoval, 1975). Furthermore, insulin may act as a pro-inflammatory agent (Garcia Leme *et al.*, 1973b; Garcia Leme *et al.*, 1974). It is interesting that adrenaline inhibits the release of insulin and stimulates the release of glucagon, both *in vitro* and *in vivo* (Malaisse, Malaisse-Lagae, Wright & Ashmore, 1967; Porte, 1967, Leclercq-Meyer, Brisson & Malaisse, 1971). Glucagon may act as an endogenous anti-inflammatory hormone that could be of therapeutic value when administered in chronic inflammation.

We are greatly indebted to Eli Lilly Labs., São Paulo, for a generous supply of crystalline glucagon and to Mr Rubens de Melo for technical assistance. This work was partly supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

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Br. J. Pharmac., (1975), 55, 69-72

REDUCTION IN THE CHOLINESTERASE ACTIVITY OF THE RAT ANOCOCCYGEUS MUSCLE PRODUCED BY CORTICOSTERONE

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1 Administration of corticosterone caused a 47% reduction in the cholinesterase (ChE) activity of homogenates of the rat anococcygeus muscle.

2 ChE activity was also reduced by morphine withdrawal and this effect was abolished by the corticosteroid synthesis inhibitor, metyrapone.

3 A single dose of reserpine reduced ChE activity in normal but not in adrenalectomized rats.

4 ChE activity was increased by adrenalectomy or by metyrapone treatment.

5 The mechanism of the corticosteroid-induced reduction in ChE activity is discussed.

6 The reduction in ChE activity produced by corticosterone, morphine withdrawal, or a single dose of reserpine might explain the leftward shift of the dose-% response curve to acetylcholine produced by these procedures in the isolated anococcygeus muscle of the rat.

Introduction

Chronic administration of corticosterone produces a characteristic pattern of supersensitivity in the rat anococcygeus muscle (Gibson & Pollock, 1973a, 1974, 1975). A similar type of supersensitivity is also produced by morphine withdrawal and by a single dose of reserpine, and in both these cases the supersensitivity appears to be linked to plasma corticosteroid levels (Gibson & Pollock, 1974, 1975), since the effects can be abolished by metyrapone, which inhibits corticosteroid synthesis, or by adrenalectomy.

Corticosteroid-induced supersensitivity in the rat anococcygeus muscle consists of two distinct components, a specific increase in the pD_2 value for acetylcholine (ACh), and a non-specific increase in the contractile response of the muscle, resulting in increased maximum responses to both noradrenaline and ACh.

The object of the present study was to investigate the mechanism underlying the first of these components. The specificity of the effect on the pD_2 value for ACh suggested that corticosterone might interfere with the metabolism of ACh, and in particular, that it might reduce the activity of cholinesterase (ChE), the enzyme of ACh degradation. Although neostigmine potentiated the response of the rat anococcygeus muscle to ACh (Gillespie & McGrath, 1974), Gillespie (1972) was unable to demonstrate the presence of the enzyme histologically. In this study, therefore, the ChE activity of the muscle was determined colorimetrically, and the effects of corticosterone, morphine withdrawal, or a single dose of reserpine on enzyme activity were investigated. In addition, the effects of the latter two procedures on animals whose adrenal function had been impaired by metyrapone or adrenalectomy respectively were studied.

Methods

Male Wistar rats (200-250 g) were killed by a blow on the head and bled from the neck. The two anococcygeus muscles were quickly dissected (Gillespie, 1972), weighed, and homogenized in 2 ml phosphate buffer (0.1 M, pH 8.0) using a Jencons 3 ml ground glass homogenizer with an electrically driven pestle. ChE activity in the homogenate was then determined by the colorimetric method of Ellman, Courtney, Andres & Featherstone (1961), with a Pye-Unicam SP 8000 dual beam spectrophotometer. All assays were performed at 37° C. The direct effect of corticosterone on ChE was determined by the addition of known amounts of the steroid directly to the assay medium. The hormone was dissolved in 50% alcohol and 32 μl of this solution was added to both test and blank cuvettes. Alcohol itself was found not to affect the assay.

Plasma corticosteroid levels were determined by a modified version of the method of Zenker & Bernstein (1958), which has been described previously (Gibson & Pollock, 1975).

Pretreatments

(i) Corticosterone (20 mg/kg, i.p. daily, for 5 days) was administered as a suspension in ethyl oleate. Control animals received appropriate volumes of ethyl oleate alone. Animals were killed on day 6.

(ii) One group of animals was given morphine (50 mg/kg, i.p. daily, for 8 days); on day 9 these rats were given a saline (0.9% w/v NaCl solution) injection and were killed on day 10. A second group received both morphine (50 mg/kg, i.p. daily, for 8 days) and metyrapone (50 mg/kg, i.p. daily, for 8 days); on day 9 they were given metyrapone alone and were killed on day 10. A third group of rats received metyrapone alone (50 mg/kg, i.p. daily, for 9 days).

(iii) Reserpine was administered in two treatment schedules. In the first, reserpine (1 mg/kg, i.p.) was administered 20 h before death. In the second, reserpine (1 mg/kg, i.p. daily) was administered for 5 days, the animals being killed on day 6.

(iv) Adrenalectomized animals were obtained commercially (Charles River Co., Margate, England), and were given saline to drink.

Drugs

The following drugs were used: acetylthiocholine iodide (B.D.H.); butyrylthiocholine iodide (Sigma); corticosterone (Sigma); metyrapone (Ciba); morphine hydrochloride (Macarthys, Glasgow), reserpine phosphate (Ciba).

Results

In Table 1 the rates of hydrolysis of acetylthiocholine and butyrylthiocholine by homogenates of the rat anococcygeus muscle are compared with those reported previously (Ellman *et al.*, 1961) for other rat tissues. It can be seen that the anococcygeus muscle exhibited a slightly greater enzyme activity than skeletal muscle tissue. Butyrylthiocholine was hydrolysed at 43%, the rate of acetylthiocholine which is in keeping with the previous finding (Ellman *et al.*, 1961). The effects of corticosterone, morphine withdrawal, and of a single dose of reserpine on the ChE activity of the anococcygeus muscle are shown in Table 2a.

Corticosterone

Administration of the steroid produced a 47% reduction in the ChE activity of the muscle.

Morphine withdrawal

ChE activity was also reduced by morphine withdrawal, although the reduction was not as great as that produced by corticosterone. Muscles from animals treated with the corticosteroid synthesis inhibitor metyrapone displayed a greater ChE activity than did controls, and this activity was not significantly altered by morphine withdrawal (Table 2b).

Reserpine

The ChE activity of the anococcygeus muscle was reduced 20 h after administration of reserpine. Muscles from adrenalectomized animals had greater enzyme activity than controls, and reserpine had no significant effect on the ChE content of muscles from adrenalectomized rats (Table 2b). Unlike a single dose, chronic administration of reserpine for 5 days did not alter the ChE activity of the muscle.

Table 1 A comparison of the rates of hydrolysis of acetylthiocholine and butyrylthiocholine in the rat anococcygeus muscles with those reported previously for other rat tissues

Tissue	*Rates (μmol hydrolysed min^{-1} g^{-1} tissue)*	
	Acetylthiocholine	*Butyrylthiocholine*
Lung[1]	1.63	0.54
Liver[1]	1.07	0.65
Muscle[1] (thigh)	1.82	0.12
Kidney[1]	0.28	0.21
Brain[1] (whole)	10.31	0.31
Anococcygeus	2.01	0.87

[1] Values taken from Ellman *et al.* (1961).

Table 2a The effect of various procedures on the cholinesterase (ChE) activity of homogenates of rat anococcygeus muscles

Treatment	*ChE activity ± s.e. (μmol hydrolysed min^{-1} g^{-1} tissue)*	n
Control	2.01 ± 0.08	32
Corticosterone (5 days)	1.07 ± 0.34*	6
Morphine withdrawal (24 h)	1 54 ± 0.21*	7
Reserpine (20 h)	1.34 ± 0.13**	8
Reserpine (5 days)	1.93 ± 0.10	6
Metyrapone (9 days)	2.56 ± 0.19*	6
Adrenalectomy	2 61 ± 0.21*	6

n = No. of observations. s.e. = Standard error of mean.
* $P < 0\ 05$; ** $0.01 > P > 0.001$—compared to control.

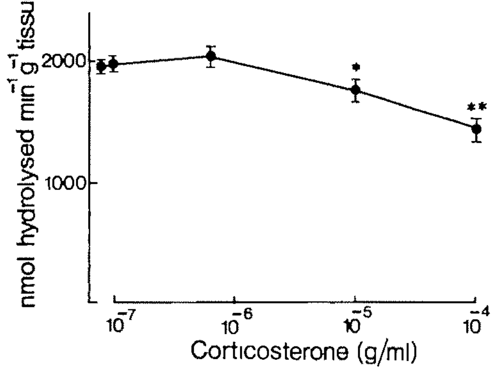

Figure 1 The effect of corticosterone on cholinesterase activity in homogenates of the rat anococcygeus muscle. The hormone was added directly to the assay medium in the concentrations shown. Each point is the mean of 6 observations and the vertical lines represent the standard errors. * $0.05 > P > 0.01$; ** $0.01 > P > 0.001$.

Comparison of plasma corticosteroid levels with concentration of corticosterone required to inhibit cholinesterase in vitro

Corticosterone inhibited the ChE activity of homogenates of anococcygeus muscles *in vitro*, at a concentration of 10 µg/ml or above (Figure 1). Examination of the plasma levels of corticosteroids in animals shown to have a reduced muscle ChE activity revealed that, although levels were raised, on no occasion were they greater than 1 µg/ml (Table 3). Plasma corticosteroids were not elevated in blood from rats treated chronically with reserpine (Table 3).

Table 2b The effect of morphine withdrawal and a single dose of reserpine on the cholinesterase (ChE) activity of muscles excised from metyrapone-treated rats or adrenalectomized rats respectively

Treatment	*ChE activity ± s.e. (µmol hydrolysed min⁻¹ g⁻¹ tissue)*	n
Metyrapone (9 days)	2.56 ± 0.19	6
Morphine withdrawal (24 h) + metyrapone (9 days)	2.78 ± 0.35	6
Adrenalectomy	2.61 ± 0.21	6
Reserpine (20 h) + adrenalectomy	3.19 ± 0.46	6

n = No of observations. s.e. = Standard error of mean.

Discussion

The present results indicate that the rat anococcygeus muscle does contain a significant amount of ChE activity, thus explaining the enhancement of the responses to ACh produced by neostigmine (Gillespie & McGrath, 1974). The rat anococcygeus muscle, therefore, provides an example of a tissue which responds to ACh and contains the enzyme of ACh degradation, but which does not appear to receive a cholinergic innervation (Gillespie, 1972).

The ChE activity of the muscle was reduced by corticosterone and was increased both by adrenalectomy and by the corticosteroid synthesis inhibitor, metyrapone. It has been suggested that the corticosteroid hormones exert a regulatory influence over the enzymes of catecholamine synthesis and degradation (Wurtman, Axelrod, Vessel & Ross, 1968, Sumathy & Clarke, 1972; Parvez & Parvez, 1972). The present results suggest that the steroids may also be important in regulating the activity of the enzyme of ACh degradation.

During morphine withdrawal, the ChE activity of the anococcygeus muscle was decreased. The involvement of corticosteroids in this reduction was indicated by the lack of effect of morphine withdrawal on the ChE activity of muscles taken from animals treated with metyrapone. This provides further indirect evidence for the possible importance of these hormones in the production of at least some of the phenomena associated with the withdrawal state (Gibson & Pollock, 1974, 1975).

The reduction of ChE activity produced by a single dose of reserpine also appeared to be due to release of excess corticosteroids since the effect was absent in adrenalectomized animals. A single dose of reserpine increases the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary, probably by stimulating the release of brain monoamines (Westermann, 1965). However,

Table 3 Plasma corticosterone levels in rats treated as shown

Treatment	*Plasma corticosterone (µg/100 ml)*	n
Control	29.7 ± 2.1	13
Corticosterone (5 days)	41.6 ± 2.7*	6
Morphine withdrawal (24 h)	45.6 ± 3.8*	6
Reserpine (20 h)	38.4 ± 3.2*	6
Reserpine (5 days)	31.1 ± 2.8	6

n = No. of observations. Values are given ± s.e. mean.
* = $P < 0.05$.

continued administration of the drug leads to a depletion of these monoamines, and hence subsequent doses of reserpine no longer stimulate ACTH release (Wells, Briggs & Munson, 1956). Indeed, it was found that chronic administration of reserpine neither raised plasma corticosteroid levels, nor reduced muscle ChE activity. Further, chronic administration of reserpine produced a pattern of supersensitivity in the anococcygeus muscle which was different from that produced by a single dose of reserpine (Gibson & Pollock, 1973b).

It is unlikely that the reduction of ChE activity was due to a direct inhibition of the enzyme by the corticosteroids, since ChE was inhibited only at high concentrations of corticosterone, which were more than ten times higher than those observed in the plasma of treated rats. The inhibition produced by high concentrations is probably due to the non-specific surface active properties of the steroids (Munck, 1957). Corticosteroid-induced reduction of enzyme activity *in vivo* is probably related to the effect of the hormones on protein synthesis and degradation (White, Blecker & Jedeikin, 1961; Parvez & Parvez, 1972).

Finally, the reduction in ChE activity of the rat anococcygeus muscle produced by corticosterone, morphine withdrawal, or a single dose of reserpine provides an explanation for the specific leftward shift of the dose-% response curve to ACh produced by these procedures (Gibson & Pollock, 1975). However, the second feature of corticosteroid-induced supersensitivity, namely the ability to increase the maximum response to agonists cannot be explained by inhibition of ChE activity, and the mechanism is at present under investigation.

This work was supported in part by the Rankin Research Fund of Glasgow University, and by the award of an M.R.C. Scholarship to A.G.

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(Received April 28, 1975.)

Br. J. Pharmac. (1975), 55, 73-78

FORMATION OF DOPAMINE AND NORADRENALINE IN RAT VAS DEFERENS: COMPARISON WITH GUINEA-PIG VAS DEFERENS

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1 The formation of [^{14}C]-3,4-dihydroxyphenylalanine (DOPA) from [^{14}C]-tyrosine, in the presence of the amino acid decarboxylase inhibitor, brocresine (3-hydroxy-4-bromobenzyloxyamine dihydrogen phosphate), was greatly enhanced in rat vasa deferentia depolarized by a KCl-enriched Krebs-Henseleit solution (52 mM KCl) compared with tissues maintained in unmodified Krebs-Henseleit solution.

2 When the conversion of tyrosine was allowed to proceed as far as catecholamine (brocresine absent) no significant difference was observed between the accumulation of [^{14}C]-catecholamines (CA) in depolarized rat vasa deferentia and the accumulation in control (non-depolarized) tissues.

3 Endogenous CA levels in the depolarized rat vasa deferentia fell to 67% of the controls after a 1 h incubation period and to 53% at the end of 2 hours.

4 Chromatographic separation on Amberlite CG-120 columns of the newly synthesized CA and catechol metabolites from the rat vas deferens revealed that a very high proportion was present as dopamine. The percentage distribution after 1 h incubation in control Krebs-Henseleit was: noradrenaline (NA)· 30.6 ± 5.2; dopamine 56.9 ± 5.9; acid metabolites: 12.8 ± 1.1; and in KCl-rich Krebs-Henseleit, NA· 32; dopamine: 44.7 and acid metabolites 23.3. In contrast to the newly synthesized (^{14}C-labelled) CA, endogenous dopamine comprises only 10% of the endogenous CA stores in rat vas deferens.

5 The distribution of newly synthesized NA and dopamine in rat vas deferens is strikingly different from that of guinea-pig vas deferens where more than 80% of newly formed amine is present as NA. In the latter tissue depolarization with K^+ causes a striking increase in CA biosynthesis.

Introduction

The ability of many sympathetically-innervated tissues to maintain endogenous stores of noradrenaline (NA) under various conditions of physiological activity arises from the combined effects of the increased formation of NA that takes place during nerve stimulation (see Weiner, 1970) and the recapture of released transmitter at the nerve ending (Iversen, 1967). If either of these processes is inhibited a rapid fall in tissue content of NA may occur. Some sympathetically-innervated tissues can, however, be partially depleted of their NA stores when subjected to prolonged nerve stimulation alone. For example, Dearnaley & Geffen (1966) were able to produce a reduction in the NA content of the spleen of the cat by stimulating the splenic nerve electrically and Chang & Chang (1965) also obtained a fall in endogenous NA in rat vas deferens by stimulation of postganglionic fibres. More recently Bisby & Fillenz (1969) reported that perfusion of the rat vas deferens with a Krebs medium containing 127 mM K resulted in a 20% decrease in the NA content of the rat vas deferens. The depletion of NA is usually only seen with prolonged stimulation at relatively high frequencies. Part of the explanation for this may lie in the fact that although the efficiency of the reuptake process is maintained over a wide range of frequencies of stimulation (Hughes, 1972) there is nonetheless a larger amount of NA released, and therefore also lost, at the higher frequencies. It has been proposed that more fibre terminals are activated at high frequencies of stimulation (Hughes, 1972). In the rat vas deferens depolarized with a K-rich medium there may also be another factor involved. In the present study we have found that there is no net increase in the accumulation of newly formed catecholamine (CA) in depolarized vasa deferentia when compared with controls even though there is an increase in the formation of [^{14}C]-DOPA from [^{14}C]-tyrosine. This is in marked contrast to the guinea-pig vas deferens where depolarization by K increases the amount of newly formed CA in the tissue by 100%. Another unusual finding in the rat vas deferens is the large proportion of newly formed CA present as dopamine rather than as NA.

Methods

Vasa deferentia dissected from rats killed by a blow on the head were incubated with [^{14}C]-tyrosine (final concentration 5×10^{-5} M; specific activity 10 mCi/mM) in Krebs-Henseleit bicarbonate medium bubbled with 95% O_2 and 5% CO_2 and maintained at 37° C or in a Krebs-Henseleit medium in which various proportions of NaCl were replaced by equimolar amounts of KCl. For most of the experiments 40% of the NaCl was replaced by KCl in the KCl-enriched medium giving final concentrations of NaCl and KCl of 70.84 mM and 52 mM respectively. The unmodified medium had the following composition (mM): NaCl 118.07, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 25.00, glucose 11.01, disodium edetate (EDTA) 0.027. At the end of the incubation the tissues were blotted rapidly, frozen on solid CO_2, weighed and homogenized in ice cold 15% trichloroacetic acid (1 to 3, w/v). The homogenate was centrifuged at 10,000 rev/min for 10 min (4° C) (Sorvall RC2B refrigerated centrifuge) and the catecholamines and catechol metabolites isolated from the supernatants by alumina chromatography (Boadle-Biber, Hughes & Roth, 1970). The incubation media were also analysed for [^{14}C]-CA and labelled deaminated metabolites by a slight modification of this procedure (Boadle-Biber & Roth, 1972a). Aliquots of the alumina eluates were used to determine endogenous NA with a modification of the fluorimetric method of von Euler & Lishajko (1961) (Boadle-Biber *et al.*, 1970), dopamine by the procedure of Laverty & Taylor (1968) with minor changes (Boadle-Biber & Roth, 1972b) and total catechol radioactivity by liquid scintillation spectrometry (see Boadle-Biber *et al.*, 1970 for details). Values for CA formation which are expressed as d min^{-1} g^{-1} fresh tissue ± s.e. mean were corrected for a recovery from the alumina of 82%. The corrections applied for endogenous NA and dopamine were 83% and 81% respectively.

In order to determine the contribution of NA and dopamine to the newly formed catechol compounds the amines isolated by alumina chromatography from two rat vasa deferentia were separated by ion exchange chromatography on Amberlite CG-120 columns (Stjärne & Lishajko, 1967). To obtain a sufficiently high level of radioactivity for the ion exchange chromatography the tissues were incubated for 1 h with [^{14}C]-tyrosine having a specific activity of 50 mCi/mM. The final concentration (5×10^{-5} M) was the same as in earlier experiments. To identify the position of the ^{14}C-catechol compounds coming off the resin column cold carriers (DOPA, NA, dopamine) were added to the PCA eluate prior to chromatography on the Amberlite resin, and their native fluorescence at 335 nm (excitation wavelength 285 nm) determined using an Aminco Bowman spectrophotofluorimeter. Radioactivity was determined by counting 1.0 ml aliquots of the pooled fractions of the acid eluate in 10 ml of a scintillation fluid (DTE, see Boadle-Biber *et al.*, 1970) using a Packard scintillation counter.

In a further experiment a comparison was made between the elution profiles of newly synthesized NA and dopamine isolated from guinea-pig and rat vas deferens. The tissues were incubated with [^{14}C]-tyrosine for 1 h in control Krebs-Henseleit solution. In the case of the guinea-pig vas deferens a specific activity of 50 mCi/mM and final concentration of tyrosine of 5×10^{-5} M was used. However, with the rat vas deferens, in order to obtain a sufficient amount of radiolabelled CA to follow the elution profiles for [^{14}C]-NA and [^{14}C]-dopamine undiluted [^{14}C]-tyrosine, 300 mCi/mM, 5 μCi/ml incubation medium and final concentration of 1.67×10^{-6} M, had to be used.

The elution profiles for the two radiolabelled amines were followed by counting 1.0 ml aliquots from each fraction (1.5 ml) of acid eluate obtained from the Amberlite column in 10 ml scintillation fluid as described earlier (Boadle-Biber *et al.*, 1970).

In one experiment the conversion of [^{14}C]-tyrosine to [^{14}C]-DOPA was followed by incubating the tissue in the presence of the aromatic amino acid decarboxylase inhibitor, brocresine, in a final concentration of 10^{-4} M. At this concentration more than 95% of the ^{14}C-catechol compounds isolated by alumina chromatography and identified by chromatography on Amberlite CG-120 was present as [^{14}C]-DOPA. The recovery of DOPA eluted from the alumina columns with 4.0 ml 0.5 N HCl, was 88%.

The specific activity of tyrosine present in the tissue at the end of the incubation was determined in some experiments using methods described earlier (Boadle-Biber *et al.*, 1970; Boadle-Biber & Roth, 1972a).

Results

The net accumulation of ^{14}C-CA from tyrosine in the rat vas deferens proceeded in a linear manner for up to 1 h in both control and 52 mM KCl Krebs-Henseleit media (Table 1a). Thereafter the net accumulation fell off in both media, but the fall was most pronounced in the K-enriched medium. No significant difference was observed in the amount of ^{14}C-CA formed and retained in control tissues and those incubated in K-rich media during the first hour of incubation (Table 1b). Increasing the proportion of NaCl replaced by KCl resulted in a drop in the content of ^{14}C-CA isolated from the tissues incubated in the K-rich media to values below that for the control, an effect which may have arisen in part from the decrease in the specific activity of the tyrosine present in these tissues (Table 2). There was an overall loss of endogenous NA with time in tissues incubated in 52 mM KCl Krebs-Henseleit medium (Table 1b) and this loss of NA was further enhanced when the proportion of KCl in the medium was increased (Table 2). No intact endogenous NA could be detected in the bath fluid, however, by the fluorimetric assay. In contrast to the finding on ^{14}C-CA synthesis, formation of [^{14}C]-DOPA was enhanced in the 52 mM KCl medium (Table 1a). This observation prompted us to identify the newly formed CA present in the tissue by Amberlite CG-120 ion exchange chromatography: CA comprised over 70% of the total labelled catechols and of this, more than half was present as dopamine (Table 3). The elution profile of the labelled CA was checked against the peak of the native fluorescence of the added carriers NA and dopamine. Its distribution was also compared with that for newly formed CA isolated from guinea-pig vas deferens; in the case of the guinea-pig 88% of the labelled catechol material was NA and only 3.5% dopamine (Figure 1). Determinations of endogenous dopamine levels in the rat vas deferens revealed that this amine comprised approximately 10% of the total tissue CA.

Table 1a Synthesis of ^{14}C-catecholamines and [^{14}]-DOPA from [^{14}C]-tyrosine in rat vasa deferentia incubated in control or 52 mM KCl Krebs-Henseleit solution

Time		*Net accumulation of ^{14}C-CA from [^{14}C]-tyrosine (d min^{-1} g^{-1} fresh tissue ± s.e. mean)*				*Net accumulation of [^{14}C]-DOPA^{+} from [^{14}C]-tyrosine (d min^{-1} g^{-1} fresh tissue ± s.e. mean)*		
(h)	n*	*Control Krebs*	n	*52 mM KCl Krebs*	n**	*Control Krebs*	n	*52 mM KCl Krebs*
0.5	6	7514 ± 1012	6	7954 ± 673	3	7297 ± 1292	3	28090 ± 4674
1.0	21	15110 ± 1067	18	16937 ± 1120	3	14235 ± 1650	3	34391 ± 4465
1.5	3	18876 ± 3185	3	14126 ± 1194				
2.0	6	21199 ± 3176	6	11536 ± 673				

Table 1b Endogenous catecholamines (CA) remaining in rat vasa deferentia after different incubation times in control or 52 mM KCl Krebs-Henseleit solution

	Endogenous CA (μg/g fresh tissue ± s.e. mean)							
	Noradrenaline				*Dopamine*			
Time (h)	n	*Control Krebs*	n	*52 mM KCl Krebs*	n	*Control Krebs*	n	*52 mM KCl Krebs*
0.5	6	9.88 ± 0.72	6	8.30 ± 0.94	—	—	—	—
1.0	22	7.81 ± 0.62	22	5.25 ± 0.40	4	1.00 ± 0.09	4	0.54 ± 0.04
2.0	6	7.30 ± 0.69	6	3.86 ± 0.42	—	—	—	—

*n = number of individual vasa deferentia analysed for ^{14}C-CA, catechol metabolites and endogenous NA and dopamine.

**n = number of individual vasa deferentia and bath fluids analysed for [^{14}C]-DOPA.

$^{+}$ Vasa deferentia were incubated in the presence of the aromatic amino acid decarboxylase inhibitor, brocresine, (10^{-4} M). More than 95% of labelled catechols were found to be present as [^{14}C]-DOPA when analysed by Amberlite CG-120 chromatography.

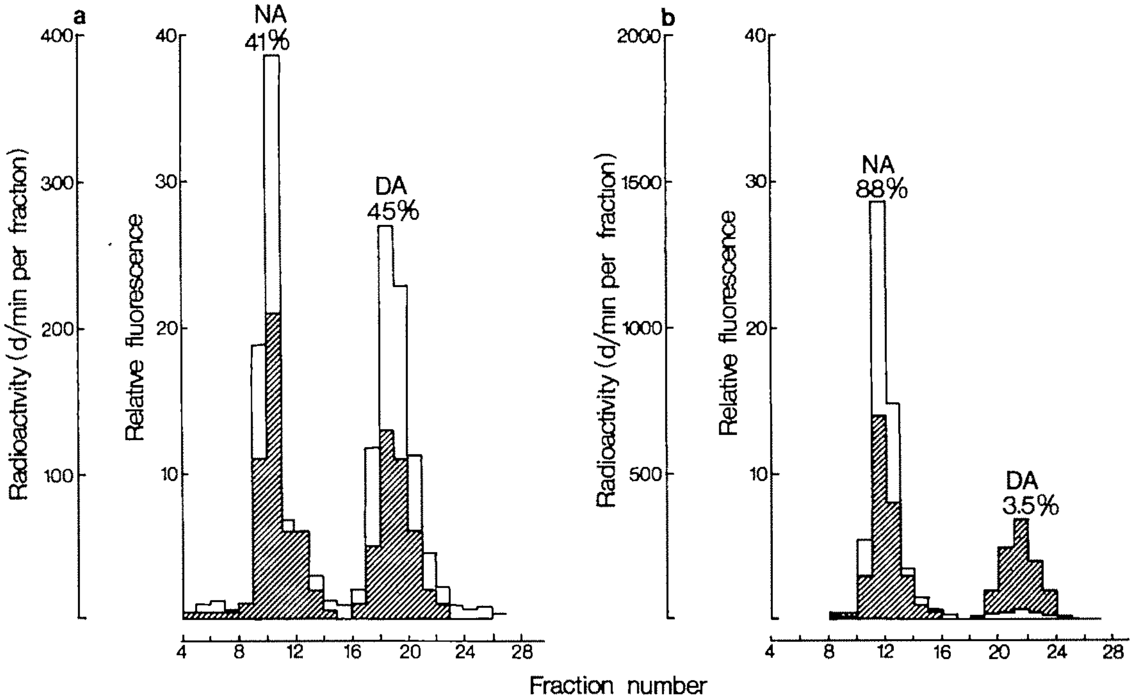

Figure 1 ^{14}C-catecholamines isolated from single pairs of (a) rat and (b) guinea-pig vasa deferentia by alumina chromatography were separated by ion exchange chromatography using Amberlite CG-120 resin according to the method of Stjärne & Lishajko (1967). Cold carriers dopamine (DA) and noradrenaline (NA) were used to identify the amine peaks by native fluorescence at 335 nm (excitation 285 nm). Open columns, radioactivity; cross-hatched columns, relative fluorescence.

Discussion

The observation that there is no net increase in the content of ^{14}C-CA or [^{14}C]-NA in depolarized rat vasa deferentia compared with controls, even though the formation of [^{14}C]-DOPA is enhanced, may be accounted for in part by preferential and rapid release of newly formed (i.e. radiolabelled) NA and in part by the accumulation of dopamine within the cytoplasm and its subsequent

Table 2 Effect of increasing concentrations of KCl on the formation of ^{14}C-catecholamines (CA) from [^{14}C]-tyrosine in the rat vas deferens incubated for 1 hour

% NaCl replaced with KCl	n*	*Net accumulation of ^{14}C-CA (d/min^{-1} g^{-1} tissue in 1 h ± s.e. mean)*	n	*Endogenous NA (µg/g fresh tissue ± s.e. mean)*	n	*Specific activity of tyrosine (d/min [^{14}C]-tyrosine/µg tyrosine ± s.e. mean)*	*Release of ^{14}C-catechol into bath^{+} (% total d/min in control)*
0	21	15110 ± 1067	22	7.81 ± 0.62	4	34932 ± 2550	100 (3627)
20	4	16431 ± 1519	4	5.07 ± 0.68	4	38601 ± 2978	120
40	18	14126 ± 1194	22	5.25 ± 0.40	4	31006 ± 1853	246
60	4	11707 ± 1617	4	3.08 ± 0.17	4	25444 ± 777	214
80	4	9305 ± 938	4	3.33 ± 0.39	4	26788 ± 1894	254

$^{+}$ Release of ^{14}C-catechols is expressed as % of the total catechol d/min present in the control media. No release of endogenous NA could be detected by fluorimetric assay.

*n = Number of individual vasa deferentia analysed.

deamination by monoamine oxidase and loss from the tissue. Analysis of the incubation media revealed that there is an increase in ^{14}C-catechol compounds present in K-rich media. However, they have not been identified and so it is not known whether they are present as intact dopamine, NA or acidic or neutral metabolites of these amines. The failure of the tissue to form or retain increased amounts of CA during depolarization clearly contributes to the net fall in endogenous amine levels seen in the vas deferens of the rat under these conditions (Table 1b; cf. Bisby & Fillenz, 1969).

The finding that such a high proportion of newly formed CA in rat vas deferens is present as [^{14}C]-dopamine is quite unexpected and is in contrast to the situation found in other sympathetically-innervated tissues where [^{14}C]-NA is the predominant CA formed from [^{14}C]-tyrosine (e.g. Alousi & Weiner, 1966; Roth, Stjärne & von Euler, 1966, 1967; Boadle-Biber *et al.*, 1970). A comparison of the proportion of [^{14}C]-dopamine found in guinea-pig vas deferens incubated in control Krebs-Henseleit medium under identical experimental conditions revealed that only 3.5% of the total catechol compounds was present as [^{14}C]-dopamine whereas 88% was in the form of NA (Figure 1). In rat vas deferens [^{14}C]-dopamine represented between 45 and 60% of the total labelled catechols. This large accumulation of [^{14}C]-dopamine which makes up more than half of the newly formed CA does not, however, reflect the contribution of dopamine to the endogenous stores of CA. Endogenous dopamine makes up only 10% of the total CA; the other 90% is present as NA

The reason for this accumulation of [^{14}C]-dopamine is not clear but a number of possibilities such as saturation of the uptake mechanism for dopamine into the storage vesicle or saturation of dopamine-β-hydroxylase, the enzyme present in the storage vesicle (Hõrtnagl, Hörtnagl & Winkler, 1969, DePotter, Smith & DeSchaepdryver, 1970) that converts dopamine to NA, seem likely. Loss of dopamine-β-hydroxylase from the vesicle during the release process (Gewirtz & Kopin, 1970; Smith, DePotter, Moerman & DeSchaepdryver, 1970; Weinshilboum, Thoa, Johnson, Kopin & Axelrod, 1971) could account for a reduction in the conversion of dopamine to NA in depolarized tissues, but does not explain the accumulation of [^{14}C]-dopamine which also takes place in rat vas deferens incubated in control Krebs-Henseleit solution.

Some caution should obviously be exercised when looking at the formation of radiolabelled CA from the amino acid precursor, tyrosine, in sympathetically-innervated tissues; any assumptions about the identity of the newly formed CA based on results from other sympathetically-innervated tissues may prove misleading.

Finally, these results may explain why it is difficult to demonstrate a depolarization-induced increase in CA biosynthesis in the rat vas deferens in contrast to the pronounced and highly reproducible acceleration of CA biosynthesis which is readily observed on depolarization of the guinea-pig vas deferens (Boadle-Biber *et al.*, 1970).

Thanks are due to Mrs Ilona Decerbo and Mr Carlis Rozitas for excellent technical assistance. This work was supported by a USPHS Grant NS 09389.

Table 3 Analysis* by Amberlite CG-120 chromatography of ^{14}C-catechol compounds formed from [^{14}C]-tyrosine in rat vasa deferentia incubated in control or 52 mM KCl Krebs-Henseleit solution.

	Control Krebs-Henseleit			*52 mM KCl Krebs-Henseleit*		
Time	*Acid metabolites*	*NA*	*Dopamine*	*Acid metabolites*	*NA*	*Dopamine*
(h)	%	%	%	%	%	%
0.5	25.5	20 1	54.4	23.8	26.0	50.1
	17.0	22.2	60.7	20.6	23.3	56.1
1.0	11.8	25.6	62.5	23.0	34.2	42.9
	11.6	25.2	63.1	23.6	29.9	46.5
	15.0	41.0	45.0			
	12.8 ± 1.1	30.6 ± 5.2	56.9 ± 5.9			

* ^{14}C-catechol compounds were isolated by alumina chromatography and then identified by Amberlite CG-120 chromatography.

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AN ELECTROPHYSIOLOGICAL ANALYSIS OF THE EFFECTS OF RESERPINE ON ADRENERGIC NEUROMUSCULAR TRANSMISSION

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1 An electrophysiological study has been made of the effects of depleting synaptic vesicles (i.e. small vesicles < 60 nm diameter) of their transmitter with reserpine on the quantity of transmitter released by nerve impulses, using the amplitude of the synaptic potential as a measure of transmitter release.

2 Pretreatment of adrenergic nerve terminals with reserpine sufficient to deplete the terminals of 70% of their noradrenaline (NA) did not change the total number of synaptic vesicles in the terminals, but did reduce the number with a large granular core as well as the quantity of NA released by a single nerve impulse by 80%.

3 Pretreatment of adrenergic nerve terminals with reserpine and iproniazid, to decrease vesicular NA but enhance cytoplasmic NA, had the same effect on synaptic vesicles and on the NA released by a single nerve impulse as did reserpine alone.

4 During a short train of impulses at high frequencies in reserpine pretreated terminals, the quantity of NA released by successive impulses increased until a steady-state release was reached comparable to that in untreated preparations. This facilitated release could be quantitatively predicted in terms of the addition of the individual potentiations introduced by each impulse in the train.

5 These results are consistent with the idea that each quantum of transmitter is stored in a synaptic vesicle, and that these may be released by nerve impulses directly from the terminal by a process of exocytosis.

Introduction

The vesicle hypothesis suggests that the structural basis for the storage and release of transmitter at synapses is the vesicle, which contains a single quantum of transmitter which is released on arrival of an impulse at a nerve terminal by the process of exocytosis (Katz, 1969; Smith, 1971, 1973). Extensive biochemical studies of adrenergic synaptic vesicles have shown that noradrenaline (NA) can be almost entirely removed from vesicles, and therefore from the nerve terminal, with reserpine (Stjarne, 1964; van Orden, Bensch & Giarman, 1967; Boadle-Biber & Roth, 1972; Enero & Langer, 1973). This procedure is known to elevate intraneuronal extravesicular NA when the enzyme monoamineoxidase is inhibited (van Orden *et al.*, 1967). However, if the synaptic vesicles in sympathetic nerve terminals are depleted of their NA content by reserpine, there is little difference in the contractile force developed by a smooth muscle during stimulation of its nerve supply with short trains of impulses (van Orden *et al.*, 1967; Ambache & Zar, 1971 Wakade & Krusz, 1972; Ambache, Dunk, Verney & Zar, 1972). In the present work an electrophysiological study has been made of the effects of altering the amount of NA in vesicles and in the intraneuronal cytoplasm on the quantity of NA released by nerve impulses.

Methods

Electrophysiology

The isolated vas deferens preparation of the mouse (Bennett, 1973) was used in all studies of adrenergic transmission. Mice were killed by cervical fracture and both vas deferens dissected free and pinned out in a perspex organ bath. Preparations were bathed in a modified Krebs-Ringer-bicarbonate solution of the following ionic composition (mM): Na 151, K 4.7, Ca 1.8, Mg 1.2, Cl 142, H_2PO_4 1.3, SO_4 1.2, HCO_3 16.3, glucose 7.8. The solution was bubbled with a gas mixture containing 95% O_2 and 5% CO_2 and maintained at 35-37° C while flowing through the organ bath at 1-3 ml/minute.

The intramural sympathetic nerves of the vas deferens were stimulated through two platinum-ring electrodes separated 1 mm apart and placed round the vas deferens, with stimuli of 50 V amplitude and 0.01-1.0 ms duration. Intracellular potentials were recorded from smooth muscle cells with glass microelectrodes filled with 2 M KCl and having resistances from 30-70 MΩ. The signals were led through a high impedance unity gain amplifier, displayed on an oscilloscope screen and photographed on moving film. The amplitude of the excitatory junction potentials (e.j.ps) in the smooth muscle were graded with the stimulus so that those potentials did not exceed 10 mV amplitude during trains of impulses, so as to avoid serious errors due to non-linear summation (Martin, 1955).

For short trains of impulses ($<$ 100) to the intramural sympathetic nerves, the amplitude of each e.j.p. was normalized with respect to the first e.j.p. in the train and the normalized data for all sympathetic nerve terminals studied were then pooled. The changes in e.j.p. amplitude during a short train were compared with predictions based on the relationship between the effect of a conditioning impulse on the amplitude of the e.j.p. evoked by a subsequent test impulse and the interval between the two stimuli derived previously (Bennett, 1973). This is given by the equation $f = f_1 \ exp - (b_1 t)$ where f is the facilitation (equal to $(V - Vo)/Vo$ where Vo is the amplitude of a conditioning e.j.p. and V is the amplitude of a test e.j.p.), and f_1 is the facilitation at zero time (equal to 0.6) and b_1 is a rate constant of decay (equal to 0.17); if the successive facilitations introduced by each impulse simply add, then the growth of facilitation during a train is described by $f = k_1 \ (1 - exp(-b_1 t))$ where $k_1 = f_1 (exp \ b_1 \Delta t - 1)^{-1}$ and Δt is the interval between successive impulses (Mallart & Martin, 1967).

Electronmicroscopy

When a vas deferens was to be examined with the electronmicroscope it was first cut into small pieces ($<$ 1 mm_3) and these were fixed in 3% glutaraldehyde in cacodylate buffer pH 7.3 for 1 h at 4-5° C and then washed overnight in 0.2 M cacodylate buffer (4.5° C). The vas deferens was then post-fixed in 2% OsO_4 in Palade's buffer pH 7.3 at 4-5° C for 2-2.5 h, rinsed briefly in tap water and stained en block with 10% uranyl nitrate solution in Palade's buffer (pH 7.3) for 1 h at 4-5° C. The vas was then dehydrated through a graded series of water-ethanol mixtures for 5-10 min and cleared in xylene. Infiltration in an Araldite mixture at room temperature for two days was followed by embedding in fresh Araldite in flat moulds; polymerization at 60° C was carried out for 1-2 days. Blocks were trimmed and silver to gold sections were cut on an LKB ultramicrotome. Sections were spread with xylene and picked up onto 200 mesh copper grids coated with a nitrocellulose film. The sections were stained on the grids with lead citrate and examined on a Phillips 200, 300 or 201 electronmicroscope. Ten or twelve electronmicrographs of adrenergic nerve-varicosity profiles were taken from one grid of sections from one block for each vas deferens examined and there was a total of 12 to 20 adrenergic varicosities in each set of micrographs; numbers of synaptic vesicles (i.e. small vesicles with diameter $<$ 60 nm) with and without a granular core (taken to be a granule with a diameter of more than 25% of the vesicle diameter) were counted for each varicosity profile, and the area of the profile determined by tracing its outline on paper, cutting out the enclosed area, weighing it and multiplying by an appropriate factor for magnification and paper weight; in this way the number of synaptic vesicles per μm^2 of varicosity profile was determined.

Drugs

Reserpine Either mice were given intraperitoneal injections of 2.5 mg/kg reserpine (Serpasil, CIBA), 48, 24 and 2 h before they were killed and the isolated vas deferens then exposed to 1.0 μM reserpine (as described by van Orden *et al.*, 1967) in the bathing medium or the vas deferens from untreated mice were exposed to reserpine 10 μg/ml in the bathing medium for 2 h before experiments started.

Iproniazid In some cases the vas deferens was exposed to this drug in the bathing medium to a final concentration of 25 μg/ml for a period of 2 h before experiments started.

Results

The effects of depleting adrenergic vesicles of noradrenaline on the quantity of noradrenaline released by a single impulse

The smooth muscle syncytium of the mouse vas deferens receives a multiple adrenergic innervation (Furness, Campbell, Gillard, Malmfors, Cobb & Burnstock, 1970; Bennett, 1972, 1973), in which

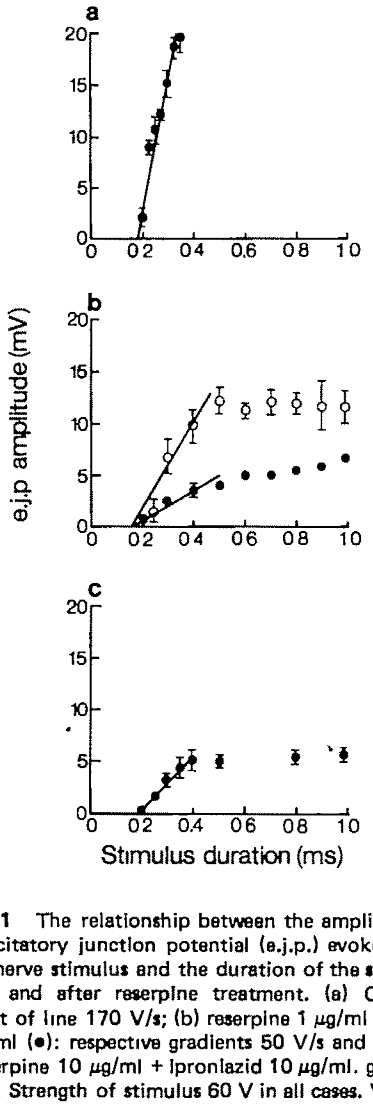

Figure 1 The relationship between the amplitude of the excitatory junction potential (e.j.p.) evoked by a single nerve stimulus and the duration of the stimulus before and after reserpine treatment. (a) Control: gradient of line 170 V/s; (b) reserpine 1 µg/ml (o) and 10 µg/ml (•): respective gradients 50 V/s and 20 V/s; (c) reserpine 10 µg/ml + iproniazid 10 µg/ml. gradient 25 V/s. Strength of stimulus 60 V in all cases. Vertical bars indicate s.e. mean for each point where it is larger than the size of the symbol; $n > 20$ from ten preparations.

the e.j.p. is reduced by over 90% by either phenoxybenzamine (50 µg/ml) or bretylium (50 µg/ml). An increase in the duration of the stimulus applied to these nerves gives an increase in the amplitude of the e.j.p. This increase in e.j.p. amplitude with stimulus duration is linear over the lowest stimulus durations (Figure 1a) up to e.j.p. amplitudes of about 15-20 mV, but over larger pulse durations (> 0.3 ms) the curve flattens off, presumably due to the effects of non-linear summation (Martin, 1955). The gradient of the linear part of the curve is 170 V/s which varies very little between cells in the one preparation or between preparations, probably because the e.j.p. in any smooth muscle cell is due to the effects of a large number of terminals releasing transmitter on other smooth muscle cells throughout the syncytium (Bennett, 1972). The gradient of this curve is proportional to the number of nerve terminals stimulated per given stimulus duration and the quantity of NA released by each stimulated terminal.

When the synaptic vesicles had been depleted of their NA by reserpine treatment, the gradient of the stimulus duration–e.j.p. amplitude curve decreased to 20 V/s (Figure 1b). At stimulus durations beyond 0.5 ms there was no further increase in e.j.p. amplitude, even though the e.j.p. was still only about 5 mV high, indicating that all the nerve terminals were now being stimulated at this duration. As it is unlikely that reserpine has any effect on the number of nerves releasing transmitter for a given set of stimulus conditions, as it has no effect on transmitter release by single impulses in the mouse phrenic nerve-diaphragm preparation (personal observations), then the reserpine effect is likely to be due to a decrease in the quantity of NA released from the nerve terminals. This decrease in the gradient of the e.j.p. amplitude-duration curve of 80% implies that reserpine has decreased the quantity of transmitter released from nerve terminals by a single impulse by this proportion.

This reserpine treatment caused a decrease of 80% in the percentage of small-diameter vesicles which had granular cores greater than one quarter the diameter of the vesicles (60.5 ± 9.9%) (30) before reserpine compared with 12.1 ± 0.5% (20) after reserpine (numbers of axon varicosity profiles studied given in brackets), without any significant change occurring in the total number of small-diameter synaptic vesicles in the nerve terminals. The similarity in the decrease in the quantity of NA released by a single impulse and in the number of vesicles with large granular cores after reserpine treatment suggests that the decrease in NA released is due to the depletion of the small vesicles of their NA content.

When NA is displaced from synaptic vesicles into the extravesicular but intraneuronal cytoplasm by reserpine, but protected from monoamineoxidase degradation by inhibition of this enzyme with iproniazid, there is an increase in the levels of cytoplasmic NA (van Orden *et al.*, 1967). However, this increase did not enhance the quantity of NA released by a single impulse over that following reserpine alone (Figure 1c). This observation implies that NA is released directly from the small synaptic vesicles following a nerve impulse, and not indirectly from the cytoplasm.

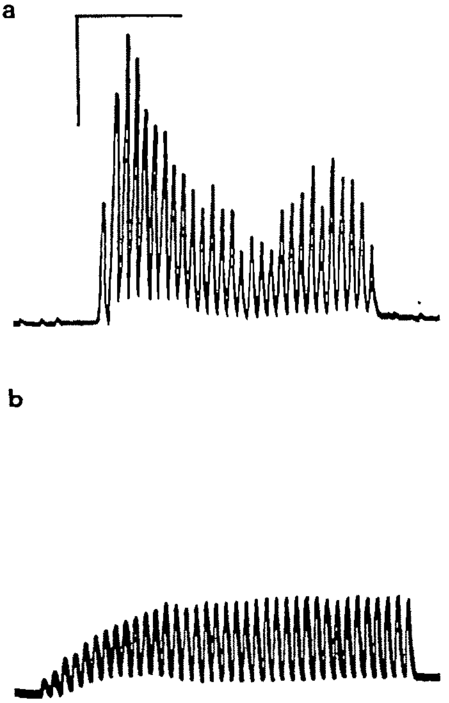

Figure 2 The effects of reserpine on the amplitude of the excitatory junction potential (e.j.p.) during short trains of high frequency impulses (10 Hz). (a) Control, (b) reserpine 10 μg/ml. Calibration: vertical, 5 mV; horizontal, 1 second.

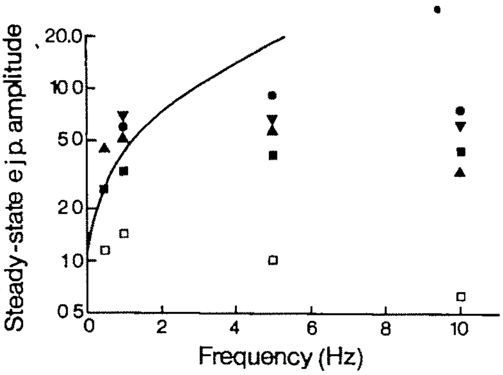

Figure 3 The effect of reserpine (10 μg/ml) and iproniazid (25 μg/ml) on the relationship between the amplitude of the steady-state excitatory junction potential (e.j.p.) reached during a short train of impulses and the frequency of stimulation. (□) Control values; (▲) reserpine *in vitro;* (▼) reserpine *in vivo* and reserpine *in vitro*; (■), reserpine + iproniazid *in vitro*; (●), reserpine *in vivo* and reserpine + iproniazid *in vitro*. The curve is the predicted relationship given in the methods. The s.e. mean was less than 15% of the mean for each point ($n > 6$).

The effect of depleting adrenergic vesicles of noradrenaline on the quantity of noradrenaline released by short trains of impulses

The amplitude of successive e.j.ps in a short-stimulus train in control preparations increases with the first few impulses and then either decreases (at high frequencies $\geqslant$ 1 Hz; Figure 2a) or continues to increase (at low frequencies < 1 Hz) until a steady-state e.j.p. amplitude is reached after a few seconds of stimulation (Bennett, 1973). The relationship between the steady-state amplitude reached during a short train of impulses and the frequency of stimulation is shown in Figure 3: the steady-state amplitude at first increases with increasing frequency, then decreases until it is similar to or less than the first e.j.p. in the train. In the presence of reserpine or after pretreatment with this drug, the amplitude of successive e.j.ps increased progressively at both high and low frequencies (Figure 2b) until a steady-state e.j.p. amplitude substantially greater than the first e.j.p. was reached after a few seconds of stimulation (Figure 3). Thus, although depletion of NA from the synaptic vesicles greatly depressed the amplitude of the e.j.p. evoked by a single impulse, repetitive stimulation at high frequencies ($\geqslant$ 1 Hz) led to a sustained facilitation of e.j.p. amplitude, such that the absolute amplitude of the e.j.p. in the steady-state was similar to that of control preparations (Figure 2). The effect of a conditioning impulse on the amplitude of the e.j.p. due to a subsequent test impulse at different intervals is shown in Figure 4: the facilitatory effect of the conditioning impulse falls into two components, one which occurs at short intervals and lasts up to 200 ms, and the other which lasts for several seconds and has a time constant of about 6 s (Bennett, 1973). Reserpine has no effect on either the short or long time course facilitation (Figure 4).

Figure 5 shows the observed growth in e.j.p. amplitude during short trains of impulses at different frequencies compared with the growth predicted on the basis that the facilitation introduced by each impulse in the train simply adds (Bennett, 1973), for control muscles and in the presence of reserpine or reserpine plus iproniazid. There is good agreement between the predicted and observed growth at 0.5 Hz (Figure 5c) in both control and drug-treated preparations, but at higher frequencies (Figure 5a, b) the control values are much smaller than those predicted whereas the treated preparations show good agreement up to 5 Hz. The normalized

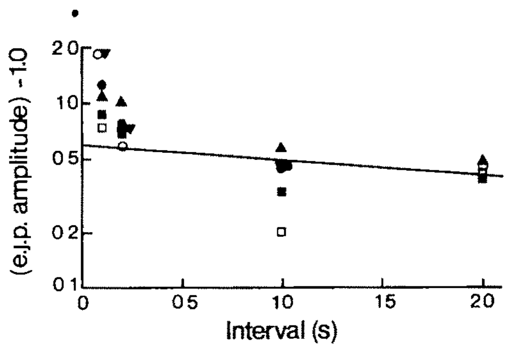

Figure 4 The action of reserpine (10 μg/ml) and iproniazid (25 μg/ml) on the effect of a conditioning impulse on the amplitude of an excitatory junction potential (e.j.p.) evoked by a subsequent test impulse. (□) and (○) Controls from two series of experiments; (■) reserpine and iproniazid *in vitro*; (●) reserpine *in vivo* and iproniazid *in vitro*; (▲) reserpine *in vitro*; (▼) reserpine *in vivo*. The s.e. mean was less than 15% of the mean for each point ($n > 6$). The line indicates a time constant of 6 seconds.

short-term steady-state e.j.p. amplitude reached at 5 Hz or 10 Hz in the presence of reserpine was about five times greater than the corresponding value in untreated preparations (Figure 3). As reserpine treatment caused about an 80% decrease in the NA released by the first impulse in a train, the NA released per impulse during the short-term steady-state in the presence of reserpine is of the same order as that in untreated preparations.

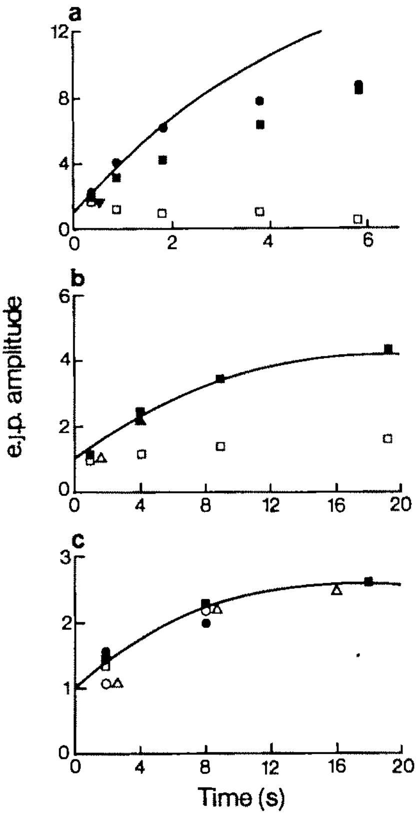

Figure 5 The effect of reserpine (10 μg/ml) and iproniazid (25 μg/ml) on the growth of the excitatory junction potential (e.j.p.) during short trains of impulses at different frequencies. (a) 5 Hz; (b) 1 Hz; (c) 0.5 Hz. (□) Control values; (●), reserpine *in vitro*; (■), (○) and (△), reserpine + iproniazid *in vitro*. Curves are the predicted relationship for the growth of the e.j.p. given in the methods section. The s.e. mean was less than 15% of the mean for each point ($n > 9$).

Discussion

The release of noradrenaline in the smooth muscle of the mouse vas deferens by impulses

The question arises as to whether the e.j.p. in response to a single nerve impulse in the mouse vas deferens is solely due to the release of NA. Although the mechanical response of the vas deferens to trains of nerve impulses is abolished by 6-hydroxydopamine (Furness *et al.*, 1970; Farnebo & Malmfors, 1971) and bretylium (Henderson, Hughes & Kosterlitz, 1972), it is possible that some transmitter is still released from nerves when preparations are exposed to these drugs, but that the e.j.p. is now reduced below threshold for initiation of the events leading to a mechanical response: thus although bretylium abolishes the mechanical response, the e.j.p. is not abolished, but is reduced to less than 10% of its control size; similarly, relatively low concentrations of phenoxybenzamine block the spontaneous contractions of the vas deferens which occur after treatment with 6-hydroxydopamine (Furness *et al.*, 1970), but they do not abolish the e.j.p., although it is reduced to less than 20% of its control size. However, when the degenerative effects of 6-hydroxydopamine on the adrenergic nerve terminals is complete, the e.j.p. is abolished (Furness *et al.*, 1970); this observation, together with the fact that the e.j.p. is unaffected by atropine (Bennett, 1973) or hyoscine (Furness *et al.*, 1970), suggests that the e.j.p. is solely due to the release of NA.

It is likely that the amplitude of the e.j.p. is a measure of the amount of NA released by a single impulse as there is good correlation between the time course of changes in noradrenaline output from sympathetic nerves during long trains of impulses (Dearnaley & Geffen, 1966; Kopin, Breese, Kraus & Weise, 1968; Stjärne & Wennmalm, 1970) and changes in the e.j.p. amplitude (Bennett, 1973).

The effect of depleting vesicles of their noradrenaline on the amount of noradrenaline released by single impulses

The vesicle hypothesis has not been directly tested at cholinergic synapses because there has not been any method available to date to differentially deplete or load vesicular acetylcholine with respect to extravesicular acetylcholine. At the adrenergic synapse, evidence to suggest that NA is released directly from synaptic vesicles into the synaptic cleft has been obtained by biochemical examination of the constituents of adrenergic vesicles and comparison of these with the constituents appearing in the perfusate from organs on stimulation of their adrenergic nerve supply. In particular dopamine-β-hydroxylase has been shown to be present in adrenergic synaptic vesicles but not free in the nerve terminal cytoplasm, and to be released on stimulation of adrenergic nerves (de Potter, de Schaepdryver, Moerman & Smith, 1969; Geffen, Livett & Rush, 1969; Chubb, de Potter & de Schaepdryver, 1970, 1972; Bisby, Fillenz & Smith, 1973). Furthermore, the soluble cytoplasmic proteins of comparable size, such as dopa-decarboxylase, are not released (de Potter *et al.*, 1969; Geffen *et al.*, 1969).

The reserpine-treatment used in the present experiments depletes the nerve terminals of over 70% of their NA content (van Orden *et al.*, 1967) which is mostly due to the removal of NA from vesicles as extravesicular NA levels are elevated by this procedure (van Orden *et al.*, 1967; Boadle-Biber & Roth, 1972) especially in the presence of monoamineoxidase inhibitors (Potter, 1967; Smith, 1972). The 80% decrease in the amplitude of the e.j.p. after reserpine is likely to be due to a similar percentage drop in the quantity of NA released by a single impulse, as reserpine has little α-adrenoceptor blocking capacity (Flemming & Trendelenburg, 1962; Trendelenburg & Weiner, 1962). Taken in conjunction with the biochemical studies this result suggests that NA is released by exocytosis from the small granular synaptic vesicles.

The effect of reserpine on the quantity of noradrenaline released by short trains of nerve impulses

The reason that the contractile response of smooth muscle to short trains of impulses (< 100 impulses at > 5 Hz) in excitatory adrenergic nerves is not depressed when the transmitter is depleted from most of the synaptic vesicles by reserpine (van Orden *et al.*, 1967; Ambache & Zar, 1971; Ambache *et al.*, 1972; Wakade & Krusz, 1972), is probably due to the growth in facilitation of the e.j.p. which occurs in the presence of reserpine compared with the usual depression which occurs in its absence (Bennett, 1973). Thus, the amplitude of the first e.j.p., which is greatly depressed by reserpine treatment, soon facilitates to control values after a few impulses. The action of reserpine may be similar to that of the α-adrenoceptor blocking drugs in promoting transmitter release, which is discussed in the following paper (Bennett & Middleton, 1975).

Because the effector response to adrenergic nerve stimulation during short trains of impulses is little affected by reserpine treatment, it has been suggested that the store of noradrenaline required for normal transmission must be extremely small (Antonaccio & Smith, 1974; van Orden, Schaefer, Antonaccio & Smith, 1974), and may be related to the small number of granular vesicles which remain in the nerve terminals after reserpine treatment (van Orden *et al.*, 1974). The present work suggests a different explanation, related to the fact that reserpine has two actions on the nerve terminal: on the one hand it depletes synaptic vesicles of their NA, leading to a decrease in the amount of transmitter released by a single nerve impulse; on the other hand reserpine alters the neurosecretory mechanisms so that increasing numbers of quanta are released by successive impulses during a short train, until the synaptic potential reaches a size comparable to that in controls.

This work was supported by the Australian Research Grants Committee.

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(Received November 1, 1974.
Revised March 24, 1975.)

Br. J. Pharmac. (1975), 55, 87-95

AN ELECTROPHYSIOLOGICAL ANALYSIS OF THE EFFECTS OF AMINE-UPTAKE BLOCKERS AND α-ADRENOCEPTOR BLOCKERS ON ADRENERGIC NEUROMUSCULAR TRANSMISSION

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1 An electrophysiological study has been made of the effects of either blocking noradrenaline (NA) uptake or α-adrenoceptors on conduction in adrenergic preterminal axons and on NA release.

2 The excitatory junction potential (e.j.p.) evoked by a single stimulus increased slightly in duration (maximum 20%) in the presence of high concentrations of desipramine or cocaine ($\geqslant$ 1 μg/ml) but there was no change in the spontaneous miniature excitatory junction potential (m.e.j.p.s); the single compound preterminal action potential was decreased in amplitude by a maximum of 10%. The e.j.p., m.e.j.p. and the terminal action potential were not altered by lower concentrations of these drugs (< 1 μg/ml).

3 The increased decline of the e.j.p. amplitude observed during the first few hundred impulses at high frequencies (10 Hz) in the presence of desipramine or cocaine was accompanied by a similar decline in the amplitude of the preterminal compound action potential, suggesting that the latter gave rise to the former.

4 These observations suggest that the action on post-synaptic α-adrenoceptors of NA released by single impulses is terminated by diffusion, and that any NA which is subsequently taken up into nerves is metabolized.

5 All the α-adrenoceptor blocking drugs tested reversed the normal depression in e.j.p. amplitude observed during the first few hundred impulses at high frequencies to facilitation; this was unaccompanied by any changes in the preterminal compound action potential.

6 α-Adrenoceptor blocking drugs did not alter the potentiating effect which a conditioning impulse had on the amplitude of the e.j.p. evoked by a subsequent test impulse. The facilitated release of NA during trains of impulses was quantitatively predicted in terms of the addition of the individual potentiations introduced by each impulse in the train.

7 It is suggested that if there is an auto-inhibition of NA release, then it is unlikely that the pre- and post-synaptic α-adrenoceptors are identical.

Introduction

Smooth muscles which receive a sympathetic innervation can take up exogenous noradrenaline (NA) (Axelrod, 1964) due to the presence of uptake processes in the membranes of both nerve (Hillarp & Malmfors, 1964; Taxi & Droz, 1966) and muscle cells (Gillespie & Muir, 1970) which may be selectively blocked by drugs (Iversen, 1963, 1965; Lightman & Iversen, 1969). During nerve stimulation much of the NA released does not diffuse from the muscle but is taken up by nerve and muscle cells where it is at least in part metabolized (Langer, 1970; Langer, Stefano & Enero, 1972; Dubocovitch & Langer, 1973). However, it is not clear whether the action of NA released by single nerve impulses onto muscle receptors is terminated by these uptake processes (Iversen, 1971) or simply by diffusion from the varicose synapses into the extracellular space of the muscle (Bennett, 1972). It is also uncertain to what extent the NA taken up by the nerve terminals during stimulation becomes available for re-release by subsequent impulses (Blakeley, Brown & Geffen, 1969; Bennett, 1973a). An electrophysiological study of these problems is described in the first part of this paper.

Phenoxybenzamine, which blocks both the

neuronal (Avakian & Gillespie, 1968) and muscle cell (Eisenfeld, Axelrod & Krakoff, 1967) uptake of NA, increases the release of NA from nerve terminals (de Potter, Chubb, Putt & de Schaepdryver, 1971; Cripps, Dearnaley & Howe, 1972) by reversing the usual depression in transmitter output per impulse observed after the first few impulses (Bennett, 1973b) to a facilitation (Bennett, 1973a). It has therefore been suggested that the NA released from nerve terminals acts on presynaptic α-receptors to in part inhibit the release of the transmitter by subsequent nerve impulses (Farnebo & Hamberger, 1971; Langer, Adler, Enero & Stefano, 1971; Kirpekar & Puig, 1971; Enero, Langer, Rothlin & Stefano, 1972; Starke, 1972; Starke & Schümann, 1972). The second part of this paper describes a study of the effects of drugs which block α-adrenoceptors on NA release.

Methods

Transmission

The isolated vas deferens of the mouse was used in all the intracellular studies of adrenergic transmission. The experimental arrangements and techniques, as well as the method of normalizing and graphing the amplitude changes of the e.j.p. during trains of impulses were the same as that described in the preceding paper (Bennett & Middleton, 1975).

Conduction

Isolated bundles of preterminal sympathetic axons (Malmfors, 1965) from the guinea-pig vas deferens were used in all studies of action potential conduction. These bundles were dissected free from the serosal surface of the vas deferens in the organ bath, at their point of entry into the superficial smooth muscle bundles (Merrillees, Burnstock & Holman, 1963); such axons are already varicose and contain synaptic vesicles (Bennett, 1972). Each end of an approximately 2 mm length of preterminal-axon bundle was taken up into fine glass-capillary suction electrodes, one of which was used to stimulate and the other to record the preterminal compound action potential.

Drugs

The following drugs were added directly to the organ bath where specified, generally to a final concentration of 10 μg/ml, and a delay of 0.5 h was allowed for equilibration before electrophysiological measurement began: desipramine hydrochloride (Geigy); cocaine; phentolamine hydrochloride (Regitine, CIBA); dibenamine hydrochloride (Smith, Kline & French); dihydroergocristine (Sandoz); dihydroergocornine (Sandoz).

Results

The effect of blocking neuronal uptake on the release of noradrenaline by a single nerve impulse

If the neuronal uptake of NA is as important for terminating the action of NA on α-adrenoceptors after its release from nerve terminals, as cholinesterase is for terminating the action of acetylcholine at the endplate (Fatt & Katz, 1951, 1952), then inhibiting neuronal uptake should greatly increase both the amplitude and time course of the

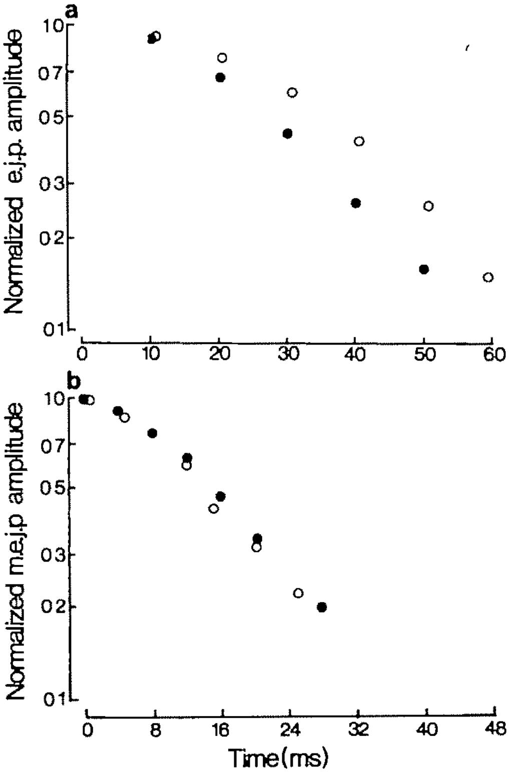

Figure 1 Time course of decline of the excitatory junction potential (e.j.p.) (a) and the m.e.j.p. (b) in the presence of amine-uptake blockers. (●), Controls; (o), desipramine (3 μg/ml). The s.e. mean was less than 5% of the mean for each point ($n > 10$)..

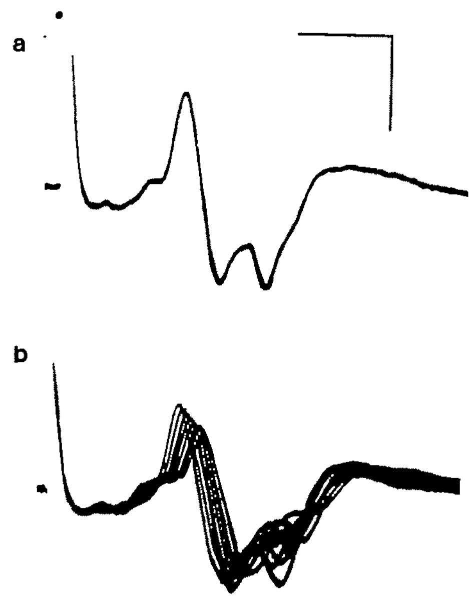

Figure 2 Change in characteristics of successive preterminal compound action potentials before (a) and during (b) exposure of a preparation to desipramine (3 μg/ml). Stimulation frequency 10 Hz. Horizontal calibration, 4 ms, vertical calibration, 100 μV.

spontaneous miniature excitatory junction potential (m.e.j.p.) due to transmitter release at close contact varicosities on smooth muscle cells (Bennett, 1972, 1973c) as well as the excitatory junction potential (e.j.p.) due to evoked transmitter release. The m.e.j.p. was unaffected by either desipramine ($\leqslant 3\ \mu$g/ml)[1] or cocaine ($\leqslant 10\ \mu$g/ml) (Figure 1b), suggesting that the action of this spontaneous transmitter release is terminated by diffusion.

The duration of the e.j.p. but not the amplitude was increased by about 20% (Figure 1a) in the presence of desipramine (3 μg/ml) and this was accompanied by a decline in amplitude and increase in duration of the preterminal compound action potential (Figure 2) of about 10% (88% ± 1.3%, $n = 6$). If this latter increase is due to the temporal dispersion of the action potentials in the preterminal axons (see below), then as the smooth muscle syncytium receives a multiple and distributed innervation from these axons (Bennett, 1972), the temporal dispersion is liable to account for the increase in duration of the e.j.p. It is unlikely that blocking neuronal uptake has simply enhanced the effect of muscle cell uptake, which then inactivates the transmitter, as blocking both neuronal uptake (desipramine 3 μg/ml) and muscle uptake (normetanephrine 10 μg/ml) simultaneously did not increase the duration of the e.j.p. over that by blocking neuronal uptake alone.

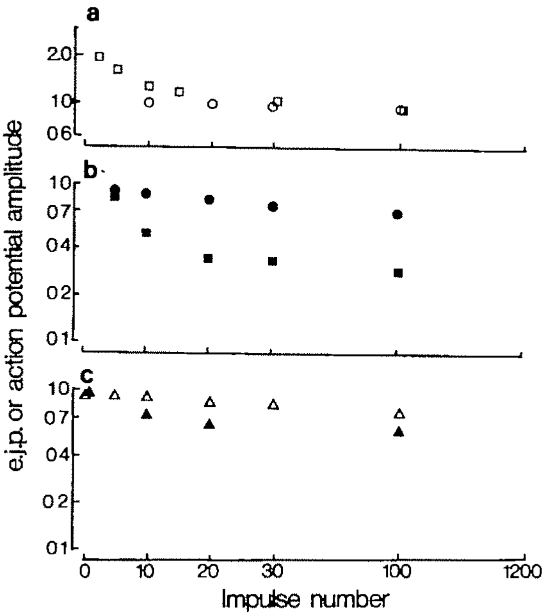

Figure 3 The decline of the compound action potential in a sympathetic preterminal nerve bundle entering the vas deferens muscle and in the excitatoty junction potential (e.j.p.) during the first 1200 impulses at 10 Hz. (a) Control (○) compound action potential, (□) e.j.p. amplitude; (b) in the presence of desipramine (3 μg/ml): (●) compound action potential, (■) e.j.p. amplitude; (c) in the presence of cocaine (10 μg/ml): (△) compound action potential, (▲) e.j.p. amplitude. Both the amplitude of the compound action potential and that of the e.j.p. were normalized to their respective values at the beginning of stimulation. The s.e. mean was less than 10% of the mean for each e.j.p. point ($n > 6$) and less than 8% of the mean for each compound action potential point ($n > 8$).

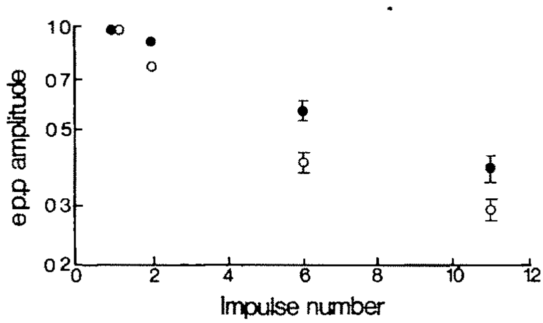

Figure 4 The decline in the amplitude of the endplate potential (e.p.p.) during the first 12 impulses at 100 Hz. (●), Control; (○), desipramine 3 μg/ml. The amplitude of successive e.p.ps is normalized to the first e.p.p. in the train. Vertical bars show s.e. mean ($n > 13$).

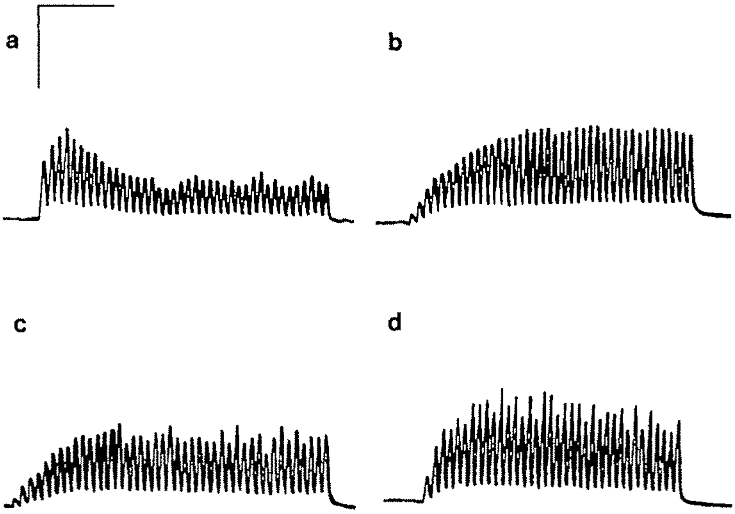

Figure 5 The effect of α-adrenoceptor blocking drugs on the amplitude of the excitatory junction potential (e.j.p.) during short trains of impulses at high frequency (10 Hz). (a) Control; (b) dihydroergocornine; (c) dibenamine; (d) phentolamine; all drug concentrations, 10 µg/ml. Calibrations: vertical, 10 mV, horizontal, 1 second.

The effect of blocking neuronal uptake on the release of noradrenaline by trains of nerve impulses

It has been reported that blocking the neuronal uptake of NA with desipramine (⩾ 3 µg/ml) or cocaine (⩾ 10 µg/ml) leads to a depression in the amplitude of successive e.j.ps in a short train (Bennett, 1973a). If it is assumed that these drugs have effects specific to their uptake blocking capacities at these concentrations, then these results suggest that normally the NA released by each impulse must be taken up and immediately re-released by subsequent impulses. This assumption has been examined by determining the effects of these drugs on the compound action potential in preterminal axons (Figure 2). Both desipramine and cocaine caused a sequential decline in the amplitude of the compound action-potential during a short train of impulses (Figure 3b and c) compared with control trains (Figure 3a) and this was accompanied by an increase in duration of the action potential. The decline reached between 30% and 50% of the control in a few hundred impulses and followed a similar time course to that of the decline in the e.j.p. (Figure 3). During continual stimulation over minutes there was no further change in the amplitude of the compound action potential following that in the first few hundred impulses, compared with controls, and the amplitude of the e.j.p. following the first few hundred impulses declined at the same rate in the presence of the drugs as in the controls (Bennett, 1973a). These observations suggest that the rapid decline in the e.j.p. during short trains in the presence of these concentrations of desipramine and cocaine is due to a decline in the nerve action potential. At lower concentrations of these drugs there is no difference in the amplitude changes, if any, in the e.j.p. or the compound action potential which accompany continual stimulation, compared with controls.

A study was made of the effects of these drugs on transmission at the endplate in order to determine whether the decline in amplitude of the compound action potential in the preterminal nerves is due to a decrease in amplitude and conduction time of the action potential in individual fibres, which would lead to a decrease in transmitter release from individual terminals (Hagiwara & Tasaki, 1958), or to complete failure of conduction in a proportion of the fibres present in the nerve (Chang & Chuang, 1972). At low frequencies (< 50 Hz) of stimulation of the phrenic nerve in the well oxygenated rat hemidiaphragm there was no difference (at the 1% level of significance) in the amplitude of the endplate potential (e.p.p.) in the presence of desipramine (3 µg/ml) compared with controls (Bennett, 1973), whereas at high frequencies (> 50 Hz) there was (Figure 4). This increased depression of the e.p.p. in the presence of desipramine was accompanied by an increase in latency, suggesting that the action of the drug is to slow conduction and decrease the amplitude of the action potential in the nerve terminal during trains of impulses, as it does the action potential in skeletal muscle (Anderson, 1973).

The effect of blocking α-adrenoceptors on the release of noradrenaline by a single nerve impulse

There was a significant decrease in the amplitude of the e.j.p. in the presence of α-adrenoceptor blockers at a concentration of 10 μg/ml (phenoxybenzamine; phentolamine; dibenamine; dihydroergocornine; dihydroergocristine), the relationship between the duration of the stimulus strength and the amplitude of the e.j.p. decreasing from a control value of 170 V/s (Bennett & Middleton, 1975) to 35 V/s, although there was no change in the amplitude of the preterminal compound action potential (see also Kao & McCullough, 1972).

The effect of blocking α-adrenoceptors on the release of noradrenaline by trains of nerve impulses

It has been reported that phenoxybenzamine reverses the normal depression in the amplitude of successive e.j.ps in a short train at high frequencies to facilitation (Bennett, 1973a). The same effect is produced by the other α-adrenoceptor blocking drugs enumerated above (Figure 5), the normal decrease in successive e.j.ps after the first few being replaced by a facilitation in which the e.j.ps continue to grow in amplitude until a steady-state NA release-rate is reached (Figure 6). There is no alteration in the amplitude of the preterminal compound action potential in the presence of α-adrenoceptor blocking agents during these changes in the amplitude of the e.j.p. occurring during short trains, so that the enhanced amplitude of the e.j.p. is presumably due to an increase in NA release from the nerve terminals.

Although the α-adrenoceptor blocking drugs greatly enhanced the steady-state e.j.p. amplitude reached during short trains of impulses (Figure 7) compared with controls, the amount by which a conditioning impulse potentiated the amplitude of the e.j.p. due to a subsequent test impulse at different intervals was approximately the same in the presence of any of the α-adrenoceptor blockers as it was in their absence (Figure 8). The amplitude and time course of decay of this facilitation following a single impulse was used to predict the growth of facilitation which occurred during trains of impulses in the presence of the α-adrenoceptor blockers (for theory, see methods section). The time course of growth of the e.j.ps during a train (Figure 6) and the steady-state e.j.p. amplitude reached at low frequencies (Figure 7) were predicted on this basis.

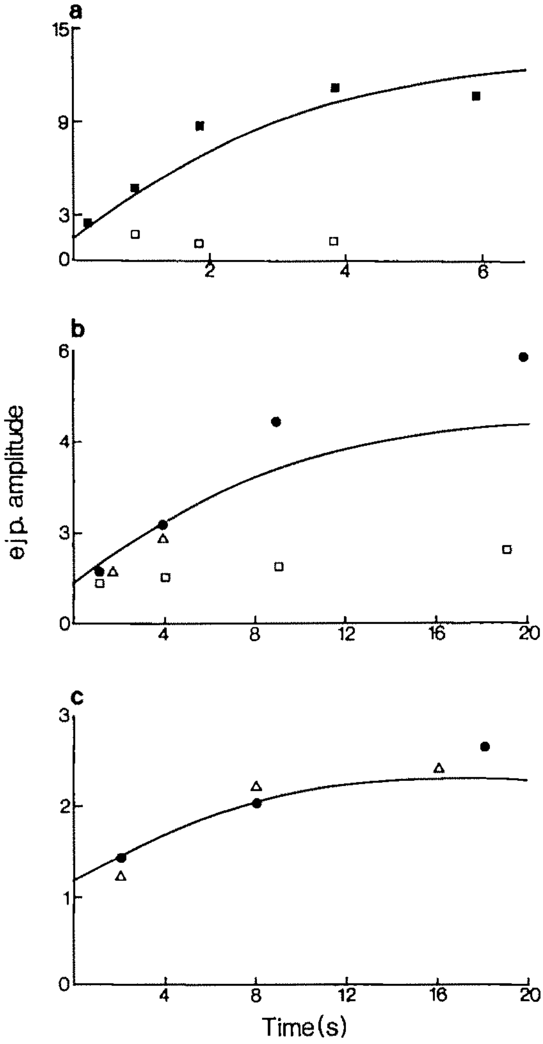

Figure 6 Effect of α-adrenoceptor blocking drugs on the growth of the excitatory junction potential (e.j.p.) during short trains of impulses at different frequencies: (a) 5 Hz; (b) 1 Hz; (c) 0.5 Hz. (●), Dihydroergocornine (10 μg/ml); (■), dihydroergocristine (10 μg/ml), (△) and (□), controls. The e.j.p. is normalized to the first impulse in the train. Curves are the predicted relationships for the growth of the e.j.p. given in Bennett (1973b). The s.e. mean was less than 20% of the mean for each point ($n > 3$).

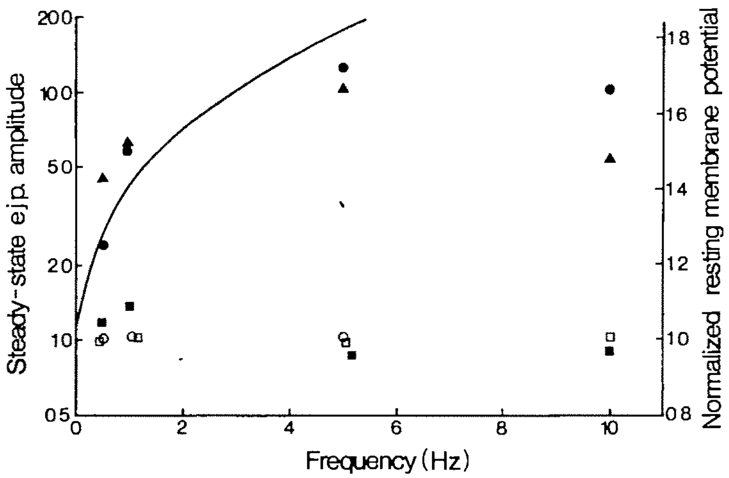

Figure 7 Effect of α-adrenoceptor blocking drugs on the relationship between the amplitude of the steady-state excitatory junction potential (e.j.p.) reached during a short train of impulses and the frequency of stimulation as well as on the resting membrane potential between successive e.j.ps when the e.j.p. steady-state has been reached. (■), (▲) and (●) are respectively the control steady-state e.j.p. amplitude, and that in the presence of dihydroergocristine (10 µg/ml) and dihydroergocornine (10 µg/ml); the steady-state e.j.p. is normalized to the first impulse in the train. (□) and (○) give the values of the membrane potential between successive e.j.ps after the steady-state e.j.p. amplitude had been reached in control and dihydroergocornine (10 µg/ml) treated preparations respectively; in each case the value of the membrane potential has been normalized to that found before stimulation commenced. The curve is the predicted relationship for the steady-state e.j.p. amplitude given in Bennett (1973b). The s.e. mean was less than 12% of the mean for each point ($n > 17$).

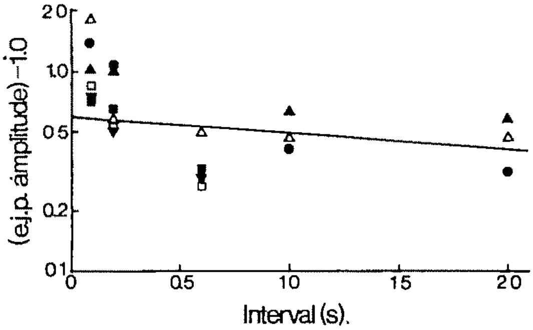

Figure 8 Effect of a conditioning impulse on the amplitude of an excitatory junction potential (e.j.p.) evoked by a subsequent test impulse at different intervals in the presence of α-adrenoceptor blocking drugs. (□) and (△), Control from two series of experiments; (■), dibenamine; (●), dihydroergocornine; (▲), dihydroergocristine; (♦), phentolamine. All drug concentrations 10 µg/ml. The ordinate scale is the test e.j.p. amplitude normalized to the conditioning e.j.p. amplitude less 1.0. The s.e.m. can was less than 10% of the mean for each point ($n > 16$). The line indicates a time constant of 6 s (see Bennett, 1973b).

Discussion

The termination of the effect of noradrenaline released by single nerve impulses

It has been suggested that amine-uptake plays a role analagous to that of cholinesterase at the motor endplate in terminating the action of the released transmitter (Iversen, 1971); however, uptake blockers have only a small effect on the e.j.p. compared with the effect of anticholinesterase on the endplate potential (Fatt & Katz, 1951, 1952). There is no change in the characteristics of the e.j.p. in the mouse vas deferens in the presence of concentrations of desipramine (1 µg/ml) or cocaine (1 µg/ml) (Holman, 1967) which are known to produce an over 60% inhibition of noradrenaline uptake (Iversen, 1965). As the preterminal action potential is unaffected at these concentrations, it is unlikely that a potentiation of the e.j.p. due to blocking NA uptake has been masked by a decrease of NA release due to changes in the preterminal action potential.

At a higher concentration of desipramine (3 μg/ml) there is about a 20% increase in the duration of the e.j.p., but little change in its amplitude, whilst cocaine produces a similar increase in duration as well as a diminution in amplitude of the e.j.p. (Holman, 1967), similar effects of cocaine have been observed on the e.j.p. of the guinea-pig vas deferens (Bell, 1966; see, however, Bennett, 1972). These drugs, even at these high concentrations, do not change the time course of the m.e.j.p. due to the spontaneous release of NA from the nerve terminals, indicating that their effect on the evoked e.j.p. is likely to be either on the nerve terminal action potential or the mechanism of evoked transmitter secretion. The observation that a single preterminal compound action potential is increased in duration with higher concentrations of drugs suggests that the slight increase in duration of the e.j.p. is due to an increase in temporal dispersion of the action potentials invading the different nerve terminals which supply the smooth muscle syncytium.

It seems likely that the NA released by a single impulse is simply inactivated by diffusing from a point of high concentration at the close-contact varicosities to a much lower concentration in the extracellular space (Bennett, 1972), as seems to be the case for acetylcholine released at preganglionic nerve terminal varicosities (Ogston, 1955, Burke, 1956). Having ceased to exert a physiological effect it may then be taken up from the extracellular space by neuronal or muscle cell uptake (Bennett, 1972), this then subserving a role with respect to NA similar to that which the cholinesterase located throughout structures in sympathetic ganglia has for the acetylcholine released within the ganglia (Bennett & McLachlan, 1972).

The effect of blocking neuronal uptake on the release of noradrenaline by trains of impulses

The increased decline in amplitude of the e.j.p. during the first few hundred impulses of a high frequency (10 Hz) train in the presence of desipramine (3 μg/ml) or cocaine (10 μg/ml) (Bennett, 1973a) is paralleled by a decline in amplitude and an increase in duration of the preterminal compound action potential, which suggests that the latter gives rise to the former. It is likely, in view of the effects of desipramine on the endplate potential, that the changes in characteristics of the sympathetic preterminal compound action potential are due to a decrease in conduction velocity and amplitude of the action potential in individual nerve fibres, and that this is the main reason for the rapid decline in the e.j.p. observed during continual stimulation in the presence of amine-uptake blockers. The alternative suggestion (Bennett, 1973a) that the rapid decline in the e.j.p. in the presence of amine-uptake blockers might be due to their uptake blocking capacity *per se* seems unlikely in the light of the present observations.

If the decline in e.j.p. amplitude in the presence of amine-uptake blockers is entirely due to effects of the drugs on the nerve impulses, then these drugs do not affect the release of NA during over 20 min of stimulation of the sympathetic nerves at 10 Hz. This suggests that any neuronal uptake of the released NA in this organ does not contribute to the subsequent release of the transmitter under these conditions.

During stimulation of more than 20% of the intramural sympathetic nerves at 10 Hz for 20 min in the presence of neuronal uptake blockers there is no change in the resting membrane potential of muscle cells between individual e.j.ps. Thus the increase in NA concentration in the extracellular spaces of the muscle during this pattern of stimulation is not sufficient to exert a physiological effect on α-adrenoceptors, either because it can quickly diffuse from this thin walled muscle (Bennett, 1972) or because it has been taken up into the muscle cells and metabolized (Dubocovitch & Langer, 1973).

The uptake of NA and its subsequent release by nerve impulses in a short train is difficult to reconcile with the vesicle hypothesis for it would require that the NA taken up in some way enhances the NA content of the vesicles which are immediately available for release, thus effectively increasing the quantal size during stimulation. The present results suggest an alternative explanation, namely that the NA taken up is metabolized.

The effect of blocking α-adrenoceptors on the release of noradrenaline by impulses

The facilitation in amplitude of the e.j.p. during the first few hundred impulses in the presence of α-adrenoceptor blockers was unaccompanied by any changes in the preterminal action potentials, indicating that these drugs were probably enhancing the release of NA from the nerve terminals (Enero *et al.*, 1972). The reversal of the normal depression in the e.j.p. during a short/high-frequency train to facilitation in the presence of the α-adrenoceptor blockers, is similar to that observed in the presence of reserpine (Bennett & Middleton, 1974); in both cases the growth of facilitation has the time course and reaches a steady-state amplitude which would be expected if the facilitation due to each impulse in the train simply adds, according to the theory previously described (Bennett, 1973b). However, the differences between the effects of the α-adrenoceptor blocking drugs and reserpine, is that the former lead to a greatly enhanced release of NA during a short train as the facilitation they introduce is not accompanied by a simultaneous depression in the quantity of NA released by the first impulse in the train (Bennett & Middleton, 1974). Nevertheless, the quantitative similarity in the facilitatory effects introduced by both the α-adrenoceptor blockers and reserpine suggests that each acts at a common point in modifying the secretory process during trains of impulses.

If the NA released by nerve impulses normally acts back on these presynaptic α-adrenoceptors located on the nerve terminal, to inhibit its own release, then it is likely that these receptors are different from the post-synaptic α-adrenoceptors. The facilitatory effect of the α-adrenoceptor blockers during a train was clear when the interval between successive e.j.ps was as great as 500 ms; during this interval the membrane potential had returned to its normal resting value (Figure 7) indicating that NA was not present in sufficient concentrations to exert a post-synaptic effect, although it is proposed that during this period it is exerting a presynaptic action. These observations can be reconciled with the suggestion that there is an auto-inhibition of NA release, if the dissociation rate constant of the presynaptic α-adrenoceptors is different from the post-synaptic α-adrenoceptors. That the two receptors are not identical is also indicated by the differencies in the potencies of phenoxybenzamine in blocking them (Dubocovich & Langer, 1974).

This work was supported by the Australian Research Grants Committee. We wish to thank Dr E.M. McLachlan for her assistance with some preliminary experiments.

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(Received November 1, 1974.
Revised March 24, 1975.)

Br. J. Pharmac. (1975), 55, 97-104

AN ELECTROPHYSIOLOGICAL ANALYSIS OF THE EFFECT OF Ca IONS ON NEUROMUSCULAR TRANSMISSION IN THE MOUSE VAS DEFERENS

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1 A study has been made of the effects of changing the external calcium concentration $[\mathrm{Ca}]_0$ and the external magnesium concentration $[\mathrm{Mg}]_0$ on the synaptic potential due to noradrenaline release.

2 When $[\mathrm{Ca}]_0$ was varied in the range 0.7 to 1.8 mM, the synaptic potential increased as about the second power of $[\mathrm{Ca}]_0$.

3 Increasing $[\mathrm{Mg}]_0$ depressed the synaptic potential; however, variation of $[\mathrm{Ca}]_0$ in the presence of high $[\mathrm{Mg}]_0$ did not significantly change the power relationship between the synaptic potential and $[\mathrm{Ca}]_0$.

4 The facilitated increase in the synaptic potential during short trains of impulses at different frequencies was quantitatively predicted on the assumption that each impulse leaves residual Ca ions bound to release receptors in the nerve terminal.

Introduction

The dependence of the evoked release of acetylcholine at the cholinergic neuromuscular junction on calcium ions has been determined from an analysis of the effects of different external calcium concentrations $[\mathrm{Ca}]_0$ on the amplitude of the endplate potential (Jenkinson, 1957). Evoked acetylcholine release increases as the fourth power of $[\mathrm{Ca}]_0$ and this action is depressed by magnesium in such a way as to suggest that the release of transmitter is mediated by the formation of a Ca-receptor complex (Ca*X*) (Dodge & Rahamimoff, 1967; Bennett, Florin & Hall, 1975). A natural consequence of this idea is that facilitation at this junction is due to a conditioning impulse leaving behind residual Ca*X* at the nerve terminal, so that the amount of Ca*X* present following a subsequent test impulse is increased, leading to an enhanced transmitter release (Katz & Miledi, 1968; Younkin, 1974).

The dependence of the evoked release of noradrenaline (NA) at the adrenergic neuromuscular junction on calcium ions has been studied by determining the NA overflow from either the spleen or vas deferens during short trains of nerve impulses in different $[\mathrm{Ca}]_0$ (Kirpekar & Misu, 1967; Stjärne, 1973); evoked NA release was shown to be dependent on about the second power of $[\mathrm{Ca}]_0$ in the range from 0.8 mM to 2.5 mM. In the present work we have examined the dependence of NA release on $[\mathrm{Ca}]_0$ and $[\mathrm{Mg}]_0$ by using the amplitude of the excitatory junction potential (e.j.p.) evoked in smooth muscle cells as a measure of NA release (Bennett, 1973a; Bennett & Middleton, 1975a); the dependence of the e.j.p. on $[\mathrm{Ca}]_0$ and $[\mathrm{Mg}]_0$ suggests that NA release is also mediated by a Ca-receptor complex (Ca*X*) and that facilitation at the adrenergic neuromuscular junction is due to the accumulation of residual Ca*X* following successive impulses.

Methods

The mouse isolated vas deferens was used in all experiments. The animals were killed with a cervical fracture, and both vasa deferentia dissected free and pinned out immediately in a Perspex organ bath of about 10 ml capacity. This was perfused with a modified Krebs solution of the following ionic composition (mM): Na 151, K 4.7, Ca 1.8, Mg 1.2, Cl 142, H_2PO_4 1.3, SO_4 1.2, HCO_3 16.3, glucose 7.8, and gassed continuously with 95% O_2 and 5% CO_2. Solutions were maintained at 34° to 36°C and flowed continuously through the bath at about 10 ml/minute. The intramural sympathetic nerves were stimulated with two platinum ring electrodes placed around the vas deferens and about 1 mm apart. Intracellular potentials were recorded from the smooth muscle cells with glass microelectrodes filled with 2 M-KCl and having resistances of 30 to 70 MΩ. The signals were led through a high impedance unity gain amplifier, displayed on an oscilloscope and photographed on moving film.

Changes in the external calcium concentration $[Ca]_0$ or external magnesium concentration $[Mg]_0$ were made by changing the level of $CaCl_2$ or $MgCl_2$ present in the reservoir of Krebs supplying the organ bath; no compensation was made for tonicity changes by altering the NaCl. The combinations of $[Ca]_0$ and $[Mg]_0$ and the number of nerves stimulated were chosen such that the e.j.p. was in general less than 10 mV, even during trains of high frequency stimulation, in order to avoid serious errors due to non-linear summation (Martin, 1955; Williams & Bowen, 1974). The derivation of the mass action equation describing the competitive actions of Ca and Mg at some point or points in the release process, together with the general method for determining the dissociation constants in the equation, are given by Jenkinson (1957) and by Dodge & Rahamimoff (1967). The derivation of the method of analysis for the residual Ca*X* following conditioning impulses is given by Linder (1973), Younkin (1974) and Zucker (1974).

At constant stimulus parameters, the amplitude of the e.j.p. at the stimulating electrode does not vary between smooth muscle cells as the synaptic potential in each cell occurs as a result of transmitter acting on cells throughout the smooth muscle electrical syncytium (Bennett, 1972); furthermore there is little difference between preparations in the amplitude of the e.j.p. recorded in cells at the stimulating electrode if the same stimulus parameters are used (Bennett & Middleton, 1975a). In the present experiments the number of nerves stimulated was kept approximately constant by stimulating with single impulses which were always of 0.5 ms duration and of 60 V strength, thus allowing the results from different cells to be pooled for any given set of ionic conditions. As the slowest component of facilitation declines exponentially with a time constant of 6 s (Bennett, 1973a), a minimum interval of 20 s was left between successive impulses when examining the effects of different $[Ca]_0$ on NA release.

Results

The effect of changes in $[Ca]_0$ on the release of noradrenaline by single impulses

The $[Ca]_0$ was changed in the range from 0.7 mM to 1.8 mM, and the amplitude of the e.j.p. due to the release of NA determined at each $[Ca]_0$. The amplitude of the e.j.p. increased with about the second power (2.3 ± 2.2 (3)) of $[Ca]_0$ over this concentration range in the presence of a constant $[Mg]_0$ (Figure 1). However, no changes in the amplitude of the m.e.j.ps were observed.

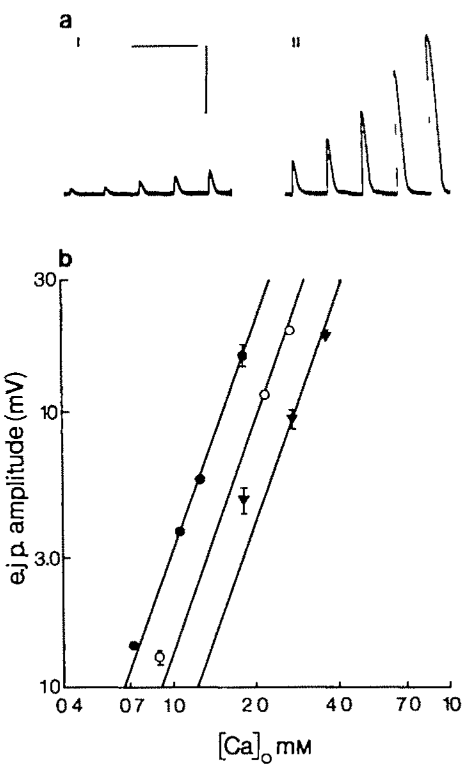

Figure 1 Dependence of the excitatory junction potential (e.j.p.) amplitude on external calcium concentrations $[Ca]_0$. (a) The effect of changing $[Ca]_0$ on the amplitude of the e.j.p. during short trains of impulses at low frequency (3 Hz); in (i) $[Ca]_0$ = 0.7 mM and in (ii) $[Ca]_0$ = 1.8 mM with $[Mg]_0$ = 1.2 mM constant; vertical calibration 20 mV and horizontal 0.6 s. (b) The effect of changing $[Ca]_0$ at three different $[Mg]_0$ is shown on log/log coordinates: (●), $[Mg]_0$ = 1.2 mM; (○), $[Mg]_0$ = 6.2 mM; (▲), $[Mg]_0$ = 11.3 mM; each point was determined from at least five e.j.ps in five cells. Vertical lines indicate ± s.e. mean for each point, where it is larger than the diameter of the point; the lines are drawn by eye and have a slope of 2.3. Stimulus parameters constant at 0.5 ms and 60 V.

As the $[Ca]_0$ is increased from 0.7 mM to 1.8 mM there is a 10 mV hyperpolarization of the vas deferens smooth muscle cells (Kuriyama, 1964; Bennett, 1967) which will itself contribute a small increase to the e.j.p. amplitude. No correction has been made for the effect of this hyperpolarization.

Increasing the $[\mathrm{Ca}]_0$ in the presence of a fixed high concentration of $[\mathrm{Mg}]_0$ produced an increase in the amplitude of the e.j.p. which again followed approximately a second power (Figure 1), although the curve was shifted to the right of that at the lower $[\mathrm{Mg}]_0$. This result is to be expected if Mg competitively blocks the action of Ca in increasing NA release at some point in the release process, as it does the release of acetylcholine.

Dissociation constants governing noradrenaline release

If Mg competitively inhibits the action of Ca in increasing the amount of NA released by nerve impulses, then it is possible to derive the dissociation constants (K_1 and K_2) in the expression describing this competitive inhibition (see Dodge & Rahamimoff, 1967, Bennett *et al.*, 1975):

$$\text{e.j.p.} = L\left(\frac{[\mathrm{Ca}]_0}{1+\frac{[\mathrm{Ca}]_0}{K_1}+\frac{[\mathrm{Mg}]_0}{K_2}}\right)^{2.3} \qquad (1)$$

where L is a constant. The constant K_2 was determined from the data in Figure 1 by considering two sets of different $[\mathrm{Ca}]_0/[\mathrm{Mg}]_0$ solutions for which the e.j.p. amplitude was maintained constant. $K_2 = 10.75 \pm 2.9$ mM (3).

Rearrangement of equation 1 to the form

$$(\text{e.j.p.})^{\frac{1}{2.3}} = \frac{L^{\frac{1}{2.3}}[\mathrm{Ca}]_0}{\left\{1+\frac{[\mathrm{Ca}]_0}{K_1}+\frac{[\mathrm{Mg}]_0}{K_2}\right\}} \qquad (2)$$

allows a double-reciprocal plot of $(\text{e.j.p.})^{-1/2.3}$ against $[\mathrm{Ca}]_0^{-1}$ to give a straight line (Figure 2). Each of these straight lines, for the three different $[\mathrm{Mg}]_0$ given in Figure 1, should according to equation 2 intercept the ordinate scale of the double-reciprocal plot at the same point, which they do (Figure 2). Furthermore each line intercepts the abscissa scale at

$$\frac{1}{[\mathrm{Ca}]_0}$$

where

$$K_1 = \frac{[\mathrm{Ca}]_0}{1+\frac{[\mathrm{Mg}]_0}{K_2}},$$

allowing three independent estimates of $K_1 = 8.61 \pm 0.31$ (3) mM. These values of K_1 and K_2 for the release of NA are greater than the equivalent values for the release of acetylcholine at the neuromuscular junction (Dodge & Rahamimoff, 1967; Bennett *et al.*, 1975), indicating that the former is less sensitive to changes in $[\mathrm{Ca}]_0$ and $[\mathrm{Mg}]_0$ than is the latter.

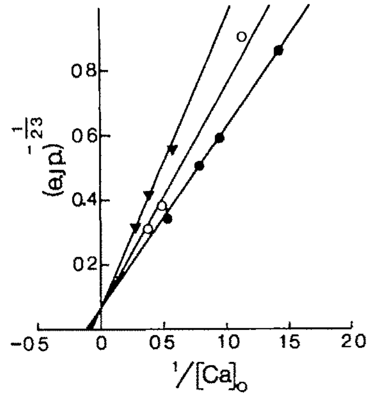

Figure 2 Double reciprocal plot for the relationship between excitatory junction potential (e.j.p.) and $1/[\mathrm{Ca}]_0$. Linear coordinates. (●), $[\mathrm{Mg}]_0 = 1.2$ mM; (o), $[\mathrm{Mg}]_0 = 6.2$ mM; (▲), $[\mathrm{Mg}]_0 = 11.3$ mM. The data shown in Figure 1 have been replotted on these coordinates and lines of best fit drawn by eye.

The effect of changes in $[\mathrm{Mg}]_0$ on the release of noradrenaline by single impulses.

In order to examine the suggestion further that $[\mathrm{Mg}]_0$ competitively inhibits the action of Ca in releasing NA, $[\mathrm{Mg}]_0$ was varied over a wide range while maintaining $[\mathrm{Ca}]_0$ constant and the changes in e.j.p. amplitude compared with the predictions of equation (1). Increasing the $[\mathrm{Mg}]_0$ decreased the e.j.p. amplitude over the entire range from 1.1 to 9.8 mM (Figure 3a); when the e.j.p. amplitude was plotted against.

$$\left(1+\frac{[\mathrm{Ca}]_0}{8.6}+\frac{[\mathrm{Mg}]_0}{10.8}\right)$$

on double logarithmic coordinates, the experimental values fell on a line of slope 2.3 as anticipated according to equation (1).

The residual CaX remaining after a single impulse

The present study has shown that the amplitude of the e.j.p. is dependent on about the second power of the external Ca concentration; furthermore, the action of Ca in releasing NA is antagonized by Mg in much the same way as is the release of acetylcholine at the endplate. The above observations suggest that facilitated release should be proportional to about the second power of

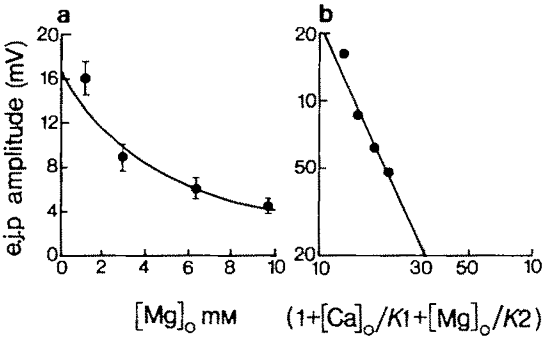

Figure 3 Dependence of excitatory junction potential (e.j.p.) amplitude on external magnesium concentration $[Mg]_o$. (a) The effect of changes in $[Mg]_o$ at a constant $[Ca]_o$ of 1.8 mM on the amplitude of the e.j.p. are shown on linear coordinates; each point was determined from at least seven e.j.ps in six cells and vertical lines indicate ± s.e. mean for each point; the line is drawn according to equation (1), with the appropriate values inserted. (b) Comparison between the predicted and observed results for the dependence of the e.j.p. amplitude on $[Mg]_o$; the data given in (a) have been replotted in (b) on log/log coordinates of e.j.p. amplitude against $(1 + [Ca]_o/K_1 + [Mg]_o/K_2)$; the line has been drawn by eye and has a slope of 2.3. K_1 = 8.6 mM, K_2 = 10.8 mM.

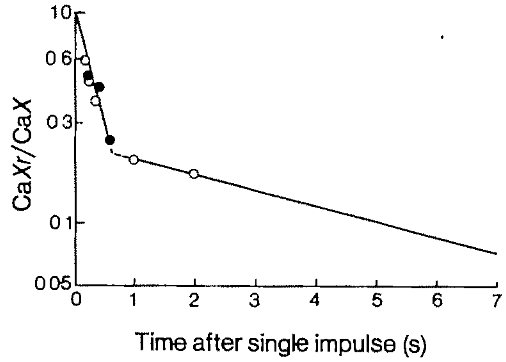

Figure 4 The decay of CaXr following a single impulse. (○) Value of CaXr determined from the decay of facilitation measured by a single test impulse at different times after a conditioning impulse; (●) value of CaXr determined from an anlysis (see text) of the facilitation which occurs during a train of four impulses at 5 Hz (see Figure 5). Straight line between 1.0 and 7.0 s, the value of CaXr determined from an analysis (see text) of the facilitation remaining after a train of four impulses at 5 Hz (see Figure 5). The decay of CaXr during the period indicated by the broken line was not determined. The s.e. mean for each (○) is less than 7% of the mean (number of observations > 17), the s.e. mean for (●) is the same as that in Figure 5.

Ca*X*. If this is so then the residual Ca*X* (Ca*Xr*) can be determined for various times after a single impulse, and used to predict the facilitated release of NA which arises as a consequence of this Ca*Xr* during trains of impulses. The residual Ca*X* at the time of a test impulse (Ca*Xr*) when expressed as a fraction of the Ca*X* formed at the time of a conditioning impulse (Ca*X*) (Linder, 1973; Younkin, 1974; Zucker, 1974) is in the present case

$$\frac{\mathrm{Ca}Xr}{\mathrm{Ca}X} = (fac.)^{\frac{1}{4}} - 1 \qquad (3)$$

where *fac.* is the ratio of the amplitudes of the test e.j.p. to the conditioning e.j.p. Figure 4 shows that at adrenergic synapses the Ca*Xr* remaining at times greater than 100 ms after an impulse disappears along two exponentials, the first with a time constant of 0.4 s and the second with a time constant of 6 s (Bennett, 1973a); this may be contrasted with the Ca*X* remaining at times greater than 100 ms after an impulse at the endplate, which also decays along two exponentials (Barrett & Stevens, 1972; Younkin, 1974), one with a time constant of about 0.3 s (Mallart & Martin, 1967; Magleby, 1973; Younkin, 1974) and a much smaller component with a time constant of several seconds (Magleby, 1973).

By analysing the growth of the facilitated release of NA which occurs during a high frequency train of impulses, and the decay of this facilitation at the end of the train (Figure 5), it is possible to estimate the Ca*Xr* remaining after different intervals following a single impulse on the basis that each impulse in the train develops the same Ca*X* and that this decays with the same time constants as for an isolated impulse (Linder, 1973; Younkin, 1974). The residual Ca*Xr* remaining from the first impulse at the time of the *n*th impulse during the train is

$$(\mathrm{Ca}Xr)_n - (\mathrm{Ca}Xr)_{n-1}$$

where $(\mathrm{Ca}Xr)_n$ equals the residual present at the time of the *n*th impulse and $(\mathrm{Ca}Xr)_{n-1}$ equals the residual present at the time of the $(n-1)$th impulse. Application of this analysis to the facilitation which occurs during four impulses at 5 Hz (Figure 5) gave values for the time course of decay of Ca*Xr* during the first 0.6 s following a single impulse similar to those previously obtained using only single conditioning-test impulses (Figure 4).

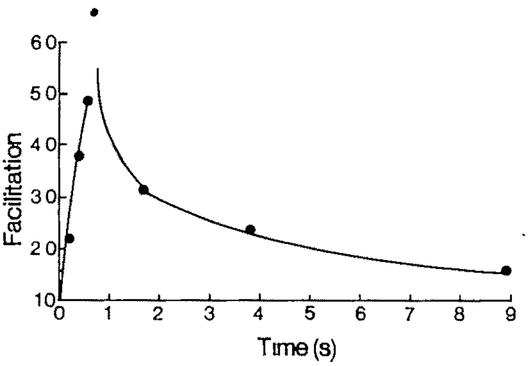

Figure 5 The facilitation of the excitatory junction potential (e.j.p.) during four impulses at 5 Hz. The decline of facilitation following the end of the train is shown for three times. The lines give the theoretical predictions for both the growth of facilitation during the train and the decline of facilitation at the end of the train, according to the theory outlined in the text. The s.e. mean is less than 10% of the mean for each point (number of observations > 10). $[Mg]_0 = 6.3$ mM, $[Ca]_0 = 1.8$ mM.

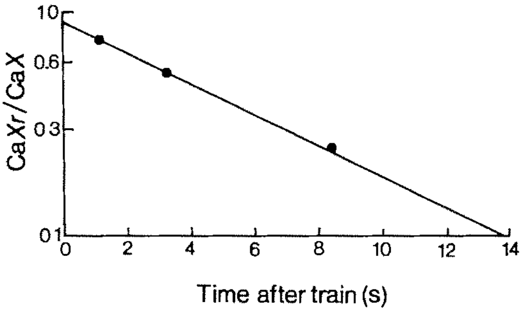

Figure 6 The decay of Ca*Xr* following a train of four impulses at 5 Hz. The straight line has a time constant of 6.0 seconds. The decay has been determined from an analysis of the data in Figure 5 (see text).

The decay of facilitation following the train of impulses in Figure 5 was determined at three different intervals, and these results used to determine the decay of Ca*Xr* after the train. As shown in Figure 6, Ca*Xr* decayed during the period from 1 to 9 s following the train along a single exponential of time constant 6 seconds. The initial Ca*Xr* component introduced by each of the four impulses which decays with a time constant of 0.4 s has completely decayed by 1 s after the end of the train, and only the cumulative effects of the second long-time course Ca*Xr* component introduced by each of the four impulses remains. It is therefore possible to use the decay of Ca*Xr* following the train of impulses (Figure 6) to determine the magnitude and time course of the slow component of Ca*Xr* introduced by each impulse. Thus for times greater than about 1 s after the train in Figure 5 (Linder, 1973; Younkin, 1974),

$$\mathrm{Ca}Xr = ce^{-(t-1.0)/\tau}(1 + e^{-\Delta t/\tau} + e^{-2\Delta t/\tau} + e^{-3\Delta t/\tau}) \qquad (4)$$

where c is the amount of Ca*Xr* remaining 1 s after a single impulse,
τ is the time constant of exponential decay,
t is the time after the end of the train,
Δt is the interval between impulses during the train.

For the train in Figure 5 and from Figure 6 $\Delta t = 0.2$ s, $\tau = 0.6$ s, and $c = 0.20$ so that $\mathrm{Ca}X = 0.20e^{-(t-1.0)/0.6}$ describes the decay of Ca*X* for periods greater than 1 s after a single impulse, as is shown in Figure 4. The slow component of decay of Ca*Xr* determined by this analysis of the decay of facilitation following a train was the same as the decay of Ca*Xr* at these times when determined by single conditioning test impulses (Figure 4).

The residual CaX remaining during and after trains of impulses

In order to test the residual Ca*X* theory for the facilitated release of NA at synapses further, the growth of facilitation during and following trains at different frequencies was predicted, using the amount of Ca*Xr* remaining at different times after a single impulse (Figure 4), and these predictions compared with experimental observations. The rise in facilitation during stimulation at frequencies between 0.5 and 7.7 Hz with three to six impulses was very well predicted (Figure 7) using the results of Figure 4, as was the decay of facilitation following these trains of impulses (Figure 7).

Discussion

The role of Ca ions in the release of noradrenaline by single nerve impulses

The e.j.p. in the mouse vas deferens is likely to be due to the release of NA as pretreatment with 6-hydroxydopamine abolishes the synaptic potential (Furness, Campbell, Gillard, Malmfors, Cobb & Burnstock, 1970) and it is reduced by over 80% with phenoxybenzamine (10 μg/ml) and bretylium

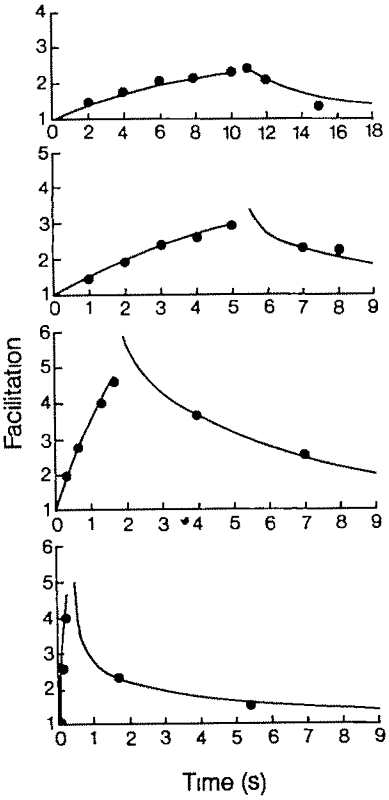

Figure 7 The growth of facilitation during trains at different frequencies, containing different numbers of impulses, together with the subsequent decay of this facilitation. From the top down the trains consist of 6,6,6, and 3 impulses at 0.5, 1.0, 3.0, and 7.7 Hz respectively. In each case these trains are followed by a test impulse triggered at various intervals to measure the rate of decay of facilitation following the train. The lines give the theoretical predictions for both the growth of facilitation during the the trains as well as the decline of facilitation following the end of the trains, according to the theory outlined in the text and the data in Figure 4. The s.e. mean is less than 10% of the mean for each point (number of observations = 5). $[\mathrm{Mg}]_0$ = 6.3 mM, $[\mathrm{Ca}]_0$ = 1.8 mM.

(10 μg/ml) but is unaffected by atropine (10 μg/ml) or hyoscine (10 μg/ml) (Bennett & Middleton, 1975a). It is likely that the amplitude of the e.j.p. is a measure of the amount of NA released per impulse as there is good correlation between the time course of changes in NA output from sympathetic nerves during long trains of impulses (Dearnaley & Geffen, 1966; Kopin, Breese, Kraus & Weise, 1968; Stjärne & Wennmalm, 1970) and changes in the e.j.p. amplitude (Bennett, 1973a), also, as shown in the present work, the e.j.p. amplitude is dependent on about the second power of $[\mathrm{Ca}]_0$ and this dependence is the same as that determined when NA overflow is used as a measure of transmitter release (Kirpekar & Misu, 1967; Stjärne, 1973).

If the internal calcium concentration at a synapse $[\mathrm{Ca}]_I$ is raised then transmitter is released (Miledi, 1973), and as the $[\mathrm{Ca}]_I$ following an impulse is momentarily raised by an amount linearly related to $[\mathrm{Ca}]_0$ (Hodgkin & Keynes, 1957; Baker, Hodgkin & Ridgway, 1971), the second power relationship between $[\mathrm{Ca}]_0$ and NA release is likely to be due to the formation of a Ca-receptor complex (Ca*X*) within the nerve terminal and not on the external surface of the terminal membrane. At the cholinergic neuromuscular junction transmitter release is governed by binominal statistics (Bennett & Florin, 1974; Bennett *et al.*, 1975), in which the quantal release parameter *n* is dependent on the third power of $[\mathrm{Ca}]_0$ whilst the probability parameter *p* is dependent on the first power of $[\mathrm{Ca}]_0$ suggesting that *n* is proportional to the third power of a calcium-receptor complex but that *p* is only dependent on the first power of a calcium-receptor complex. As the structural basis of the binomial statistical parameters is not known, it is not clear if the same calcium-receptor complex is likely to control both *n* and *p*, or whether this calcium-receptor complex resides in the terminal membrane or wholly within the nerve terminal (Cooke, Okamoto & Quastrel, 1973). It is not possible to test if NA release at the adrenergic neuromuscular junction obeys binomial statistics because of the syncytial couplings between the smooth muscle cells (Bennett, 1972), so that it is not known if the second power relationship between NA release and $[\mathrm{Ca}]_0$ arises as a consequence of the sensitivity of the parameters *n* and *p* on the formation of Ca-receptor complex nor whether this complex resides in the terminal membrane or inside the terminal or both.

The antagonism by Mg of the action of Ca in increasing NA release by an impulse observed in the present study, and in a previous study by Kirpekar & Misu (1967) using NA overflow as a measure of release, does not help to determine the site at which Ca-receptor complexes are formed in the nerve terminal. Mg antagonizes the entry of Ca into axons which accompanies the nerve impulse (Baker *et al.*, 1971) as well as itself entering the axon during the impulse (Baker & Crawford, 1972). Thus the effects of Mg observed in the present study could be due to this ion blocking Ca movement through the membrane or entering the terminal or both.

The role of Ca ions in the release of noradrenaline by trains of nerve impulses

The facilitation of NA release by successive impulses during and following a short train can be successfully predicted on the basis that each impulse leaves residual Ca*X* in the nerve terminal in an amount determined from a study of the potentiating effects of a conditioning impulse on a test-impulse, and that transmitter release is proportional to the second power of Ca*X*. At low frequencies (< 1 Hz) there is less than 10% difference between the predictions of this theory based on a second power relationship and a linear relationship (Bennett, 1973a), so that it is not possible to distinguish experimentally between the two. However, after a few impulses at high frequencies (> 2 Hz) there is a greater than 30% difference between the predictions for facilitation based on a second power relationship and a linear one, and it is easy to distinguish that the second power relationship (Figure 7) gives the better fit to the experimental observations.

During a short train of high frequency impulses, the amplitude of the e.j.p. increases with successive impulses at the beginning of the train and then decreases until a depressed steady amplitude is reached (Bennett, 1973a). In the present work the first few facilitated e.j.ps during high frequency trains were predicted on the basis of the accumulation of residual Ca*X* but this hypothesis does not readily provide an explanation for depression; a similar change in the amplitude of synaptic potentials during short high frequency trains is observed at the cholinergic neuromuscular junction (Mallart & Martin, 1968), where the depression is attributed to a growing depletion in the number of quanta of transmitter which are readily available for release. It might be argued that the depression observed at the adrenergic neuromuscular junction is due to a growing α-adrenoceptor mediated autoinhibition of NA release, in which the NA released by an impulse acts on the nerve terminal to decrease the entry of Ca during a subsequent impulse, this more than counteracting the effects of residual Ca*X* (Stjärne, 1973). Reserpine (Bennett & Middleton, 1975a) as well as the α-adrenoceptor blockers (Bennett, 1973b; Bennett & Middleton, 1975b), reverse the normal depression which develops during a short high frequency train to a facilitation, so that it is unnecessary for a drug to possess an α-adrenoceptor blocking capacity in order to relieve this depression. If both types of drug act at a common point in the release process to reverse depression to facilitation it is possible that they both act within the nerve terminal to prevent the depletion of quanta that are readily available for release by nerve impulses.

We should like to thank Miss J Stratford for technical assistance. This work was supported by the Australian Research Grants Committee.

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(Received April 17, 1975.
Revised June 5, 1975.)

Br. J. Pharmac. (1975), 55, 105-110

INTERACTION OF MONO- AND DIVALENT METALLIC CATIONS AND OF INDOMETHACIN ON THE MEMBRANE POTENTIAL OF VASCULAR ENDOTHELIAL CELLS *in vitro*

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1 Endothelial cells depolarized progressively when heated for 5 h at 45° C in the presence of calcium (1 mM), cupric (0.08 mM) or ferrous (0.04 mM) ions. In the absence of these ions, heating caused only slight depolarization. Higher concentrations of these ions caused depolarization even at normal body temperature (37° C).

2 Cuprous and potassium ions, although producing depolarization at 37° C, failed to augment the depolarization due to heating to 45° C.

3 Hydrogen peroxide caused depolarization which was potentiated by the presence of calcium, cupric or ferrous ions, but not by the presence of cuprous or potassium ions.

4 Indomethacin (0.25 mM) reduced the depolarization caused by calcium, cupric or ferrous ions at 37° C and also reduced the potentiation of heat-induced and hydrogen peroxide-induced depolarization which these divalent metallic cations produced. However, indomethacin failed to modify the depolarization caused by cuprous or potassium ions.

Introduction

An earlier study revealed that vascular endothelial cells possess a resting membrane potential which may be measured *in vitro* during exposure of the vessel wall to bathing fluids of known composition (Northover, 1975b). Heating the vessel wall to 45° C, or exposing it to histamine, depolarized the endothelial cells if the bathing fluid contained calcium ions but had no effect in the absence of calcium. Several anti-inflammatory drugs reduced the depolarization observed at 45° C in the presence of calcium. The experiments to be described in this paper were designed to explore further the inter-relationships between the effects of certain metallic cations, and of various types of physical and chemical injury, on the membrane potential of vascular endothelial cells.

Methods

Membrane potentials of endothelial cells from the thoracic aorta of guinea-pigs were recorded as described previously in detail (Northover, 1975b). The isolated aorta was cut longitudinally and opened out into a sheet which was then tied to the bottom of a superfusion trough. Glass micropipettes filled with 3 M KCl solution and having a resistance of approximately $10^7\,\Omega$ were lowered vertically by means of a micromanipulator towards the endothelial surface of the aorta. A recording was considered to begin with the first negative change of voltage encountered, and this was assumed to originate from the interior of the surface layer of cells. The pipette was allowed to remain within a cell for 50 s and then withdrawn into the superfusate. The difference in recorded voltage just before and just after withdrawal was taken as the membrane potential. The tip-potentials of the micropipettes were measured whenever the composition of the superfusate was altered, by the method of Adrian (1956). A micropipette was discarded and replaced by a new one if the tip-potential exceeded 10 mV. This problem was rare except when superfusates containing a low concentration of chloride ions were used. Except where otherwise stated, the experiments were conducted at 37° C in a basic superfusion fluid (BSF) of the following composition (mM): NaCl 130, KCl 6, glucose 11, $MgCl_2$ 2, tris-(hydroxymethyl)-methylamine 10, adjusted to pH 7.0 with acetic acid and oxygenated by a stream of air bubbles.

Results

Interaction between the effects of sodium, potassium, chloride, magnesium and calcium ions on the response to heating

Endothelial cells depolarized progressively during 5 h of heating to 45° C in a bathing fluid consisting of the BSF to which calcium chloride (1 mM) had been added (Table 1). Depolarization was very slight when the cells were heated in BSF to which the metal-chelating agent 1,2-bis-2-aminoethoxyethane-*NNN'N'*-tetraacetic acid (EGTA) had been added (Table 1). Exposure to a temperature of 37° C for 5 h caused no significant change in membrane potential in the absence or in the presence of calcium chloride (1 mM).

Replacement of all the NaCl in the BSF by an equiosmolar concentration of choline chloride (130 mM) or of sucrose (260 mM) increased the heat-induced depolarization observed in the presence of added calcium chloride but not in its absence (Table 1). Replacement of most of the chloride ions in the BSF by an equimolar concentration of the larger and less permeant benzenesulphonate ions had no significant effect on the magnitude of heat-induced depolarization either in the presence or in the absence of calcium ions (Table 1).

Omission of potassium or of magnesium ions from the bathing fluid or the addition of ouabain (10^{-4} M) to the BSF had no significant effect on the magnitude of heat-induced depolarization in the presence of calcium ions (Table 2).

Table 1 Effect of various ions on the membrane potential of endothelial cells during heating to 45° C for 5 hours

Composition of superfusate (mM)					*Membrane potential (mV negative)**					
Sodium chloride	*Sodium benzene sulphonate*	*Choline chloride*	*Sucrose*	*Calcium chloride†*	*At 37° C***	*During heating to 45° C. Duration (h)*				
						1	*2*	*3*	*4*	*5*
130	0	0	0	0	40 ± 5	40 ± 4	37 ± 6	38 ± 6	36 ± 5	35 ± 4§
130	0	0	0	1	36 ± 6	27 ± 6	25 ± 5	23 ± 6	18 ± 5	15 ± 4‡
0	130	0	0	0	38 ± 7	36 ± 6	35 ± 4	34 ± 6	35 ± 7	36 ± 5§
0	130	0	0	1	37 ± 6	30 ± 5	26 ± 4	22 ± 4	17 ± 4	14 ± 3
0	0	130	0	0	42 ± 5	39 ± 8	40 ± 6	39 ± 5	36 ± 5	38 ± 6§
0	0	130	0	1	30 ± 4	19 ± 4	18 ± 3	12 ± 3	9 ± 2	6 ± 2§
0	0	0	260	0	48 ± 6	42 ± 8	40 ± 6	39 ± 5	36 ± 5	37 ± 6§
0	0	0	260	1	31 ± 5	22 ± 4	16 ± 4	10 ± 3	7 ± 3	4 ± 1§

* The means of at least 30 observations ± s.e. mean. **Values recorded after 1 h exposure to a superfusate of the stated composition. †Where no calcium chloride was added, the BSF contained EGTA 0.1 mM.
‡ A significant difference exists between the value marked ‡ and values marked § (Student's *t* test, $P < 0.05$).

Table 2 Effect of substances which may alter sodium-pump mechanisms on the membrane potential of endothelial cells during heating to 45°C for 5 h in the presence of calcium chloride (1 mM)

Composition of superfusate (mM)				*Membrane potential (mV negative)**				
Ouabain	*Potassium chloride*	*Magnesium chloride*	*At 37° C***	*During heating to 45° C. Duration (h)*				
				1	*2*	*3*	*5*	*5*
0	6	2	36 ± 6§	27 ± 6	25 ± 5	23 ± 6	18 ± 5	16 ± 4†
0.1	6	2	33 ± 4π	29 ± 6	28 ± 6	26 ± 7	20 ± 5	14 ± 3‡
0	0	2	34 ± 7π	31 ± 7	30 ± 5	25 ± 6	19 ± 6	15 ± 5‡
0	6	0	39 ± 5π	26 ± 5	23 ± 4	21 ± 5	17 ± 4	13 ± 5‡

* The mean of at least 30 observations ± s.e. mean. ** Values recorded after exposure for 1 h to the stated conditions.
† No significant difference exists between the value marked † and the values marked ‡ (Student's *t* test, $P > 0.05$), or between the value marked § and the values marked π.

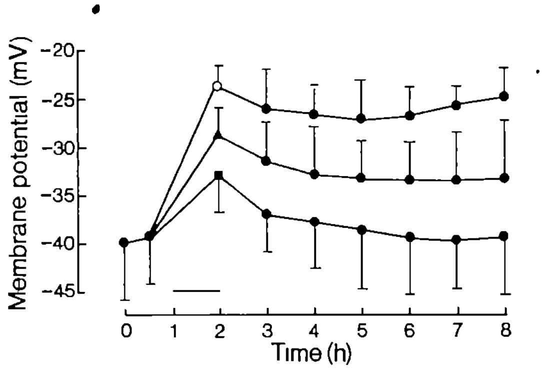

Figure 1 Membrane potential of endothelial cells in basic superfusion fluid (BSF) (●). The BSF contained added $CaCl_2$ for the period marked with a horizontal bar in a final concentration of 5 mM (■), 10 mM (▲) or 20 mM (○). Each point is the mean of at least 30 observations. Vertical bars show s.e. mean.

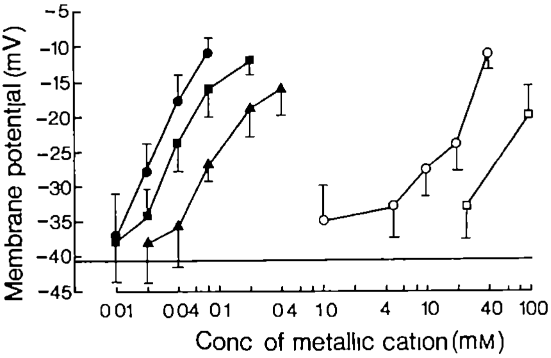

Figure 2 Membrane potential of endothelial cells exposed for 1 h to basic superfusion fluid (BSF) to which CuCl (●), $FeCl_2$ (■), $CuCl_2$ (▲), $CaCl_2$ (○) or KCl (□) had been added. Each point is the mean of 30 observations. Vertical bars show s.e. mean. The horizontal line at —41 mV is the membrane potential of cells exposed to the normal BSF.

Comparison of the depolarizing influence of calcium, cuprous, cupric, ferrous and potassium ions

Calcium ions depolarized endothelial cells when added to BSF at 37°C (Figure 1). To varying extents, this effect was shown also by potassium, ferrous, cuprous and cupric ions (Figure 2). In view of the previously reported ability of indomethacin to reduce the depolarizing effect of calcium ions (Northover, 1975b), it was of interest to establish whether indomethacin would also reduce the depolarizing effect of these other metallic cations. Indomethacin (0.05 and 0.25 mM) failed to alter significantly the depolarizing effect of added potassium or cuprous ions but reduced the influence of exposure for 1 h to added cupric or ferrous ions (Figure 3).

Concentrations of calcium insufficient to cause significant depolarization at 37°C, nevertheless enhanced the depolarization caused by heating to 45°C (Table 3). In a similar way, the divalent cations of iron and copper, in concentrations which produced a slight depolarization at 37°C, enhanced the depolarization due to heating to 45°C (Table 3). In contrast, the monovalent

Table 3 Effect of various ions on the membrane potential of endothelial cells during heating to 45°C for 5 hours

Additions to the BSF				*Membrane potential (mV negative)**				
Metallic chloride	*Concentration (mM)*			*During heating to 45°C. Duration (h)*				
	Metallic chloride	*Indo-methacin*	*At 37°C***	*1*	*2*	*3*	*4*	*5*
Control	—	0	41 ± 5	40 ± 5	41 ± 6	36 ± 5	37 ± 4	34 ± 6†
Calcium	1	0	35 ± 4	28 ± 5	22 ± 4	20 ± 3	16 ± 2	14 ± 3‡
Calcium	1	0.25	38 ± 5	39 ± 6	36 ± 5	31 ± 4	29 ± 4	28 ± 5
Potassium	24	0	34 ± 6	34 ± 5	33 ± 4	32 ± 5	30 ± 6	30 ± 5
Potassium	24	0.25	36 ± 5	32 ± 6	32 ± 5	33 ± 6	32 ± 5	34 ± 6
Cuprous	0.02	0	29 ± 5	30 ± 5	28 ± 5	26 ± 5	24 ± 4	25 ± 4
Cuprous	0.02	0.25	30 ± 5	28 ± 5	27 ± 6	25 ± 4	26 ± 4	26 ± 5
Cupric	0.08	0	27 ± 4	26 ± 5	20 ± 4	15 ± 3	13 ± 2	12 ± 3‡
Cupric	0.08	0.25	29 ± 5	29 ± 4	30 ± 6	31 ± 4	34 ± 5	33 ± 6
Ferrous	0.04	0	26 ± 4	20 ± 4	15 ± 3	15 ± 2	10 ± 3	11 ± 3‡
Ferrous	0.04	0.25	31 ± 6	30 ± 6	31 ± 5	28 ± 3	28 ± 5	29 ± 4

* The means of at least 30 observations ± s.e. mean. ** Values recorded after 1 h exposure to a superfusate of the stated composition.

† A significant difference exists between the value marked † and the values marked ‡ (Student's *t* test, $P < 0.05$).

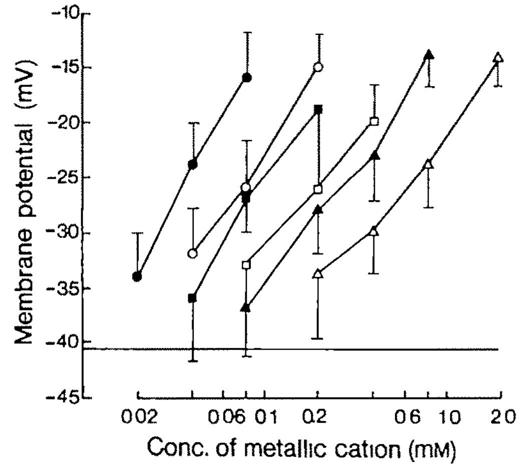

Figure 3 Membrane potential of endothelial cells exposed for 1 h to basic superfusion fluid (BSF) to which $FeCl_2$ (●), $CuCl_2$ (■), $FeCl_2$ plus indomethacin 0.25 mM (▲), $FeCl_2$ plus indomethacin 0.05 mM (○), $CuCl_2$ plus indomethacin 0.25 mM (△), or $CuCl_2$ plus indomethacin 0.05 mM (□) had been added. Each point is the mean of 30 observations. Vertical bars show s.e. mean. The horizontal line at −41 mV is the membrane potential of cells exposed to the normal BSF.

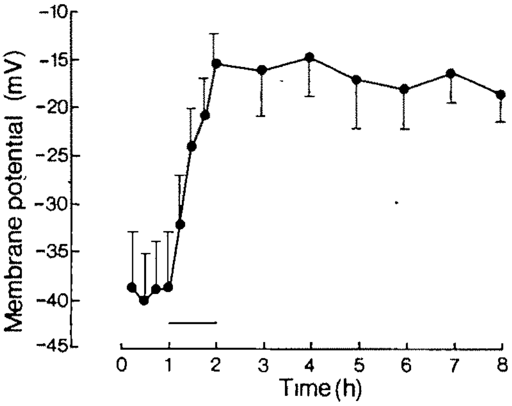

Figure 4 Membrane potential of endothelial cells. The basic superfusion fluid contained added hydrogen peroxide (120 mM) for the period marked with the horizontal bar. Each point is the mean of 30 observations. Vertical bars show s.e. mean.

Table 4 Interactions between various metallic ions and hydrogen peroxide on the membrane potential of endothelial cells

Metallic chloride	*Additions to the BSF*			*Membrane potential (mV negative)**			
	Concentration (mM)			*Duration of exposure to superfusate (min)*			
	Metallic chloride	*Hydrogen peroxide*	*Indomethacin*	*15*	*30*	*45*	*60*
Control	—	0	0	39 ± 6	38 ± 5	41 ± 5	40 ± 5†
Control	—	30	0	36 ± 5	36 ± 7	34 ± 5	35 ± 4
Control	—	30	0.25	38 ± 5	37 ± 4	36 ± 6	34 ± 5
Control	—	120	0	32 ± 6	24 ± 5	19 ± 2	15 ± 4‡
Control	—	120	0.25	33 ± 6	22 ± 4	18 ± 4	14 ± 3‡
Calcium	1	0	0	34 ± 5	30 ± 6	32 ± 6	35 ± 7
Calcium	1	30	0	29 ± 5	30 ± 5	21 ± 4	15 ± 3‡
Calcium	1	30	0.25	36 ± 4	37 ± 5	35 ± 6	36 ± 6
Potassium	24	0	0	35 ± 7	36 ± 5	32 ± 6	33 ± 5
Potassium	24	30	0	34 ± 5	32 ± 4	33 ± 7	32 ± 4
Potassium	24	30	0.25	33 ± 6	30 ± 6	29 ± 3	31 ± 7
Cuprous	0.02	0	0	34 ± 6	30 ± 6	32 ± 5	28 ± 5
Cuprous	0.02	30	0	32 ± 7	31 ± 6	30 ± 6	27 ± 3
Cuprous	0.02	30	0.25	35 ± 7	34 ± 5	29 ± 5	26 ± 4
Cupric	0.08	0	0	30 ± 6	27 ± 5	24 ± 4	27 ± 4
Cupric	0.08	30	0	31 ± 5	23 ± 4	20 ± 3	11 ± 2‡
Cupric	0.08	30	0.25	35 ± 6	32 ± 5	30 ± 6	31 ± 6
Ferrous	0.04	0	0	34 ± 5	31 ± 5	28 ± 6	24 ± 4
Ferrous	0.04	30	0	28 ± 4	24 ± 4	17 ± 3	10 ± 4‡
Ferrous	0.04	30	0.25	36 ± 7	29 ± 4	33 ± 5	32 ± 6

* The means of at least 30 observations ± s.e. mean.

† A significant difference exists between the value marked † and the values marked ‡ (Student's *t* test, $P < 0.05$).

cations of copper and potassium failed to promote heat-induced depolarization (Table 3). Indomethacin protected against the enhancement of heat-induced depolarization caused by added calcium, ferrous and cupric ions (Table 3).

Interactions between the depolarizing effects of metallic cations and of hydrogen peroxide

Exposure of endothelial cells to various concentrations of hydrogen peroxide in BSF for 1 h at 37°C caused progressive depolarization, as shown in Table 4. Depolarization was only slightly reversed on returning the tissue to the original BSF after exposure to hydrogen peroxide (Figure 4). No reduction in the concentration of sodium chloride in the BSF was made to compensate for the addition of hydrogen peroxide. The permeability of endothelial cell membranes to hydrogen peroxide is not known and hence the extent to which the addition of this substance to the BSF induced an osmotic change across the plasma membrane of these cells is also not known. However, if an osmotic pressure was developed it is unlikely to have influenced the recorded membrane potential since the addition (100 mM) to the BSF of either lithium chloride or sodium chloride produced no significant change in membrane potential in an earlier study (Northover, 1975b).

The depolarization caused by hydrogen peroxide was augmented by the presence of calcium, cupric or ferrous ions but not by the presence of cuprous or potassium ions (Table 4). The combined effect of exposure for 1 h to hydrogen peroxide plus either cupric or ferrous ions was greater than the sum of the effects of these depolarizing agents separately (Table 4).

Indomethacin failed to reduce the depolarizing effect of hydrogen peroxide in BSF but reduced the larger depolarization caused by a combination of hydrogen peroxide with either calcium, cupric or ferrous ions (Table 4). Indomethacin reduced the effects of such combinations of depolarizing agents to that obtained with hydrogen peroxide in BSF. Indomethacin failed to modify the depolarizing effect of a combination of hydrogen peroxide with either cuprous or potassium ions (Table 4).

Discussion

The concentration of calcium ions normally present in mammalian extracellular fluids (approximately 1 mM) was sufficient to sensitize vascular endothelial cells to the depolarizing effect of heat or of hydrogen peroxide (Tables 1 & 4). In the case of heat-induced depolarization the effect of calcium ions was decreased by an opposing effect of the sodium ions (approximately 130 mM) present in most mammalian extracellular fluids and in the BSF (Table 1). This calcium-opposing effect of sodium ions was not shared by magnesium, potassium, choline, chloride or benzenesulphonate ions or by sucrose (Tables 1 & 2). Sodium ions compete with calcium ions for the occupation of binding sites in several tissues (Daly & Clark, 1921, Luttgau & Niedergerke, 1958; Bohr, Seidel & Sobieski, 1969; Sitrin & Bohr, 1971). The ability of sodium ions to oppose the depolarizing effect of calcium ions on heated endothelial cells, therefore, is probably due to competition between the two cations for occupation of a cellular binding site which is important for the regulation of the membrane potential.

A lack of extracellular potassium or magnesium, or the presence of ouabain, impedes the function of the sodium-pump and leads to depolarization in several tissues (Casteels, 1966; Matthews & Sutter, 1967; Bolton, 1973). Depolarization did not occur, however, in the present experiments to any greater extent than in the BSF (Table 2). It is not known whether the membrane potential of these cells is independent of a sodium-pump mechanism or whether the pump is insensitive to ouabain and to a lack of potassium or magnesium.

Indomethacin reduced or prevented the depolarizing action of the divalent metallic cations but did not influence that of the monovalent metallic cations or of hydrogen peroxide. Furthermore, the ability of the divalent cations of calcium, copper and iron to potentiate heat-induced and hydrogen peroxide-induced depolarization was inhibited by indomethacin. It would appear, therefore, that indomethacin reduces the action of divalent but not of monovalent metallic cations on a component of the cell which is important for the regulation of membrane potential. Further work is required to identify more precisely the nature and location of this site.

It is not yet known whether the binding of naturally occurring endogenous divalent metallic cations, such as calcium, to cellular membranes promotes or permits the increases in the permeability of vascular walls which accompanies the inflammatory response. It is of interest, however, that several exogenously administered divalent metallic ions produce an increased permeability of the small blood vessels to plasma proteins (Miles & Wilhelm, 1955; Steele & Wilhelm, 1967; Mariano, Lourdes, Moraes & Neto, 1969). Endothelial cells exposed to histamine exhibit several morphological and histochemical changes which have been shown recently to be augmented by the presence of calcium and to be inhibited by indomethacin (Northover, 1975a). The possibility clearly exists, therefore, that the anti-inflammatory action of indomethacin involves an effect of the drug on the binding of divalent metals to endothelial cell membranes.

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(Received March 31, 1975.
Revised May 7, 1975.)

Br. J. Pharmac. (1975), 55, 111-116

MECHANISM OF MECHANICAL INHIBITION OF SMOOTH MUSCLE BY OUABAIN

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1 Ouabain (10 μg/ml) caused an initial increase followed by a decrease in tension in guinea-pig taenia coli bathed in normal sodium and potassium containing medium.

2 The excitatory effect of ouabain was prevented by the elevation of extracellular potassium while the inhibitory effect was abolished in the absence of sodium in the bathing medium.

3 In the presence of sodium, the inhibitory effect of ouabain disappeared when the drug was washed out, but in the presence of lithium (which is not extruded by the Na-K pump) the inhibition was not abolished.

4 The inhibitory effect of ouabain was accompanied by an increase in tissue Na and a decrease in tissue K concentration. The increase in intracellular [^{45}Ca] which occurred when extracellular [K] was varied, was also reduced by ouabain.

5 It is concluded that a high intracellular level of sodium inhibits contractions in visceral smooth muscle probably by a mechanism involving decreased Ca influx.

Introduction

The effect of cardiac glycosides on smooth muscle is inconsistent. In some multi-unit vascular smooth muscles ouabain produces an increase in tension (Cushny, 1925; Leonard, 1957; Brockaert & Godfraind, 1973) but in single unit smooth muscle various workers (Schatzmann & Ackermann, 1961, Godfraind & Godfraind De Becker, 1963; Daniel, 1964; Matthews & Sutter, 1967; Griffin, Szaro & Weltman, 1972) have found that initial excitation is followed by inhibition. The mechanism of the inhibitory action of ouabain is not clearly understood. In the present paper an attempt has been made to test whether inhibition of the sodium pump by ouabain is responsible for its inhibitory effect on the guinea-pig taenia coli. A preliminary account of this work has already been published (Bose, 1974).

Methods

Adult guinea-pigs of either sex were exsanguinated after stunning. Strips of taenia coli (resting length of 2.5 cm) were dissected out and suspended in a 'normal salt solution' (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.4, $MgSO_4$ 1.2, $CaCl_2$ 2.5, Tris-HCl 10, glucose 11. The solution was bubbled with O_2 and the pH adjusted to 7.3 by addition of 10 N HCl. The strips were attached to Grass FT-03C force displacement transducers and subjected to a resting tension of 0.5 grams. The strips were made to contract by adding KCl (124.1 mM) to the bathing medium (hypertonic KCl medium). In some experiments all the NaCl was replaced by an equimolar amount of KCl (Na-free KCl medium). 'Hypertonic Na-free KCl medium' was made by replacing NaCl with 236 mM sucrose and adding 118 mM KCl. 'Li-medium' was made by replacing NaCl with 118 mM LiCl and adding 118 mM KCl.

Uptake of sodium by tissues was measured in two different ways:

(a) Strips were mounted on stainless steel holders and incubated in the normal salt solution for 1 h at a resting tension of 0.5 g, 0.6 μCi of ^{22}Na (New England Nuclear Corp.) was added to the medium and at 15 min intervals the tissues were taken out and rinsed for 2 s twice in non-radioactive medium. Radioactivity was immediately counted for 1 min in a γ spectrometer and the tissues were then returned to the radioactive medium. The experiment was arranged so that the tissues never stayed outside the bathing medium for more than 90 seconds. Some drying of the tissue was inevitable but parallel experiments showed that this did not appreciably affect the reactivity of the muscle to KCl or to ouabain. After the ^{22}Na content of the tissues reached a steady level the solution was replaced by hypotonic KCl medium. After 15 min ouabain was added to the bath and the ^{22}Na level in the tissue was measured at 15 min intervals for 30 minutes. After the tissues were returned to the normal ^{22}Na medium recovery from the effect of ouabain was observed every 15 min for 45 minutes. In parallel experiments the effect on tension was observed. The radioactivity results were expressed as a ratio of ct $\text{min}^{-1}\,\text{g}^{-1}$ tissue wet weight: ct $\text{min}^{-1}\,\text{ml}^{-1}$ medium.

(b) Tissue content of Na and K was measured by flame photometry. Inulin space was measured after incubating the tissues for 30 min with inulin-[methoxy ^{14}C] (New England Nuclear Corp.). The tissues were blotted and weighed and the inulin was extracted by boiling in 1 ml distilled water for 20 min in a polypropylene test tube. A glass marble placed over the mouth of the test tube prevented loss of water vapour. An aliquot of this aqueous extract was mixed with scintillation fluid (Bray, 1960) for measuring radioactivity due to inulin and the rest was used for measuring Na and K. Aliquots were also taken from the incubation media for measuring radioactivity and Na and K concentrations. Total-issue water was determined by first measuring the wet weight and then measuring dry weight after drying *in vacuo* for 12 h at 80° C. The intracellular concentrations have been expressed as mmol/litre intracellular water and were calculated according to Boyle, Conway, Kane & O'Reilley (1941).

^{45}Ca uptake was measured by first incubating three sets of tissues in the normal salt solution (phosphate omitted). One group of tissues was then maintained in the normal medium. The other two groups of tissues were incubated in 'hypertonic KCl medium' (phosphate omitted) and one of these was also exposed to ouabain. ^{45}Ca (3 µCi/ml) was added to all the media and after 45 min 10 mM lanthanum chloride was added. After 5 min all muscles were then transferred to fresh medium of the same composition except that it lacked calcium and was non-radioactive. Phosphate was omitted in order to avoid interaction with lanthanum. The tissues were blotted, weighed and solubilized with NCS (ICN). Radioactivity in the tissues and in an aliquot of the medium was measured by liquid scintillation counting. Results are expressed as ratio of ct $\text{min}^{-1}\,\text{g}^{-1}$ tissue wet weight: ct $\text{min}^{-1}\,\text{ml}^{-1}$ of medium. This method gives an approximate estimate of intracellular Ca and is based on the assumption that lanthanum displaces all the extracellular Ca as well as trapping the intracellular Ca (van Breeman, Farinas, Gerba & McNaughton, 1972). Errors may result from intracellular penetration of lanthanum and a differential effect on influx and efflux of Ca, but these errors are probably similar in ouabain-treated and normal tissues and have been assumed not to invalidate comparisons between the two.

Ouabain (Nutr. Biochem. Corp.) was made up freshly as a 10 mg/ml solution in distilled water and the final concentration in the bath was always 10 µg/ml.

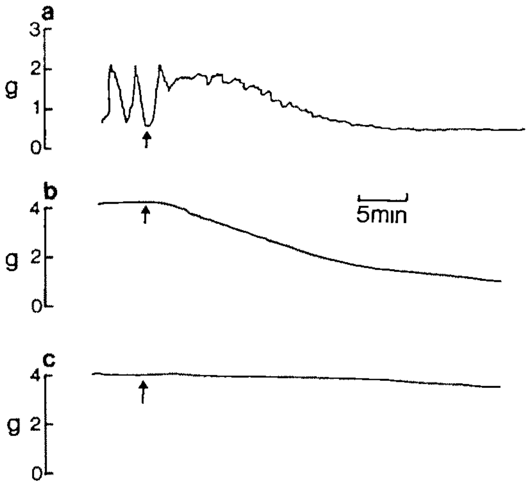

Figure 1 Isometric tension in guinea-pig taenia coli. Ouabain 10 µg/ml added at arrows. (a) Response in normal sodium and potassium containing medium. (b) Response after 15 min exposure to solution containing high potassium (KCl 124.1 mM) and normal sodium. (c) Response in presence of high potassium (KCl 124.1 mM) after exposure to sodium-free (replaced by sucrose) medium for 30 minutes.

Statistical analysis of the data was done by either the paired of the unpaired *t*-tests (Steel & Torrie, 1960).

Results

Effect of ouabain on resting tension

Ouabain (10 µg/ml) caused an initial increase in the tension of spontaneously active strips of guinea-pig taenia coli in the normal salt solution. The peak increase in tension due to ouabain (3.75 ± 0.3 g; $n = 10$) was similar to the peak amplitude of a spontaneous contraction. The increase in tension due to ouabain reached a maximum in 5 min and then gradually subsided in 15 min, and could be distinguished from the much briefer (< 2 min) duration of spontaneous contractions (Figure 1a).

Effect on depolarized taenia

Increasing the external potassium concentration to 124.1 mM without altering normal sodium concentration (hypertonic KCl medium) resulted

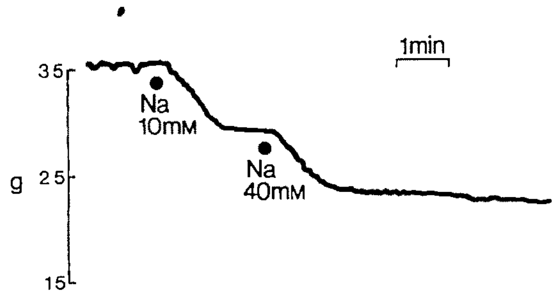

Figure 2 Effect of increasing sodium concentration to 10 and 40 mM (as indicated) on a muscle exposed to Na-free KCl medium and ouabain (10 μg/ml).

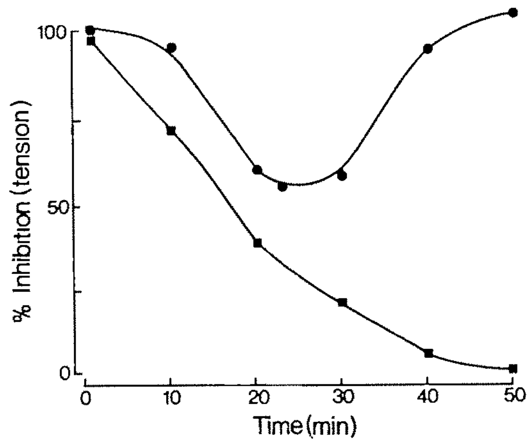

Figure 3 Effect of washing out ouabain (10 μg/ml) with normal sodium medium (●) or sodium-free lithium substituted medium (■) (both containing 124.1 mM KCl) on tension in taenia coli exposed to a sodium-free (sucrose substituted) medium containing 124.1 mM KCl for 30 min followed by ouabain for 20 minutes.

in a sustained increase in tension (4.45 ± 0.25 g; $n = 8$). Addition of ouabain then caused a gradual decrease in tension to 12 ± 3.2% of the pre-ouabain level in 32 ± 3 minutes. The depolarized taenia did not exhibit any initial increase in tension when ouabain was added, in contrast to the normally polarized preparation (Figure 1b). When ouabain was washed out in the presence of the hypertonic KCl medium the tension returned to the previous level in 45-90 minutes.

Similar experiments were done with Na-free KCl medium. This solution also caused a sustained increase in tension of the taenia to a level similar to that in the hypertonic KCl medium. Addition of ouabain caused the tension to decrease only to 68 ± 6% ($n = 8$) of the pre-ouabain level, which was a significantly smaller effect than occurred in the presence of sodium in the medium ($P < 0.01$). Similar results were obtained in a hypertonic Na-free KCl medium (Figure 1c). Exposure to the Na-free media for less than 20 min failed to inhibit the action of ouabain. These results indicate that the stimulant effect of ouabain was not seen in the depolarized muscle whereas the inhibitory effect required the presence of extracellular sodium.

Effect of restoring sodium or substitution with lithium

Taenia coli strips were first exposed to Na-free KCl solution for 30 min as described in the preceeding section. The presence of ouabain in the bath for 30 min had a very small effect. If NaCl was then added to the bath cumulatively to attain concentrations of 10 and 40 mM, the tension decrease was 12.5 ± 0.5% and 20 ± 1.8% ($n = 6$) respectively (Figure 2). The relaxation seemed to occur more rapidly than that due to ouabain in the K-depolarized or normal preparation. This could be because the effect of ouabain on the sodium pump was already established before restoration of sodium.

Since the inhibitory effect of ouabain occurred only in the presence of extracellular sodium it seemed possible that the inhibition caused by ouabain was due to intracellular accumulation of sodium. To test this hypothesis the following experiments were done:

Eight taenia strips were first exposed for 30 min to hypertonic Na-free KCl medium and then ouabain was added. After 20 min, by which time the normal effect of ouabain is established the ouabain was washed out and the bathing media of 4 strips were replaced by hypertonic KCl medium (containing Na) and of the remaining strips by the lithium medium. The muscles exposed to hypertonic KCl medium relaxed at first but after 25 min the tension returned towards normal. The muscles kept in lithium medium also relaxed but their tension never recovered (Figure 3). Similar results were also obtained if the muscles were exposed to the lithium medium without prior exposure to ouabain ($n = 3$). This was expected because lithium is not pumped out of the cell after it gets in. On the other hand, sodium is expected to be pumped out of the cell as ouabain is washed out and the Na-K pump recovers.

Effect on ionic composition of taenia

^{22}*Na influx* Six strips of taenia coli were incubated for 30 min in the normal salt solution

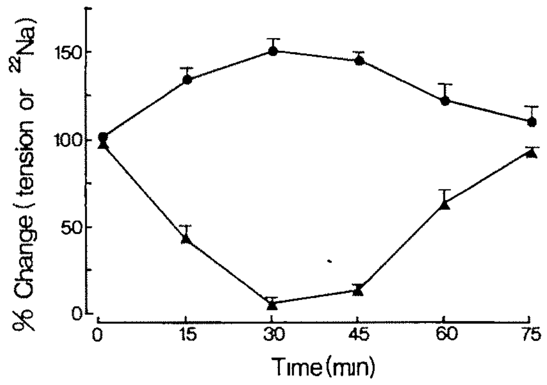

Figure 4 Effect of ouabain (10 μg/ml) on total tissue ^{22}Na (●) and tension (▲) in taenia coli exposed to hypertonic-KCl medium and ^{22}Na for 30 minutes.

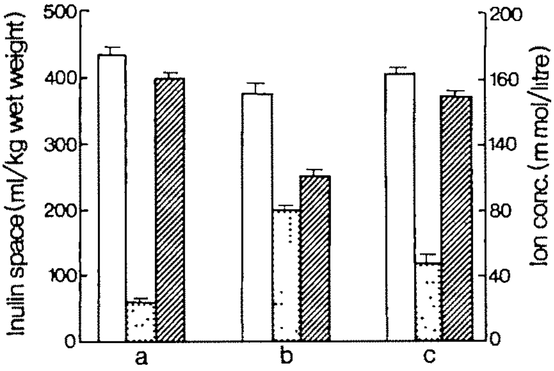

Figure 5 Effect of ouabain on inulin space (open columns), intracellular [Na] (stippled columns) and [K] (cross-hatched columns) concentrations of taenia coli exposed to the normal medium. (a) Control. (b) 30 min after ouabain (10 μg/ml). (c) 30 min after ouabain (10 μg/ml) followed by removal of ouabain for 45 minutes.

containing ^{22}Na. Na exchange was essentially complete by this time.

The KCl concentration was now increased to 124.1 mM. On adding ouabain to the bathing medium the ^{22}Na content increased to 130 ± 6.3% and 152 ± 6% of control after 15 and 30 min respectively. At the same time the tension declined to 43.8 ± 6.4% and 6.3 ± 2.5% of control respectively. After washing ouabain out the ^{22}Na level decreased to 146.9 ± 4.4%, 123.7 ± 8.8%, and 113.3 ± 10% after 15, 30 and 45 min respectively. The corresponding tensions were 15 ± 2.5%, 65 ± 7.5% and 96.52 ± 1.9% of control (Figure 4).

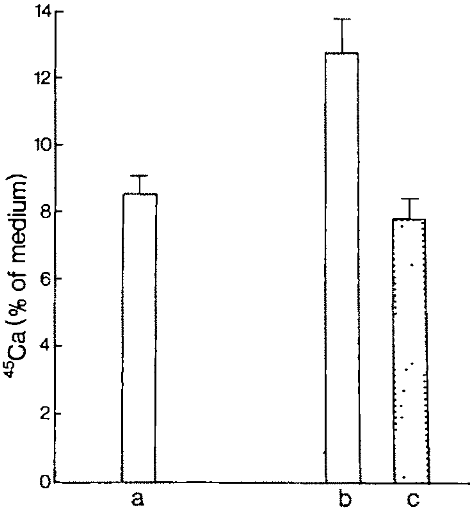

Figure 6 Intracellular accumulation of ^{45}Ca in taenia coli exposed to (a) normal, (b) hypertonic KCl medium, or (c) hypertonic KCl + ouabain, 10 μg/ml.

Intracellular Na and K concentrations Eighteen strips of taenia coli were incubated in the normal salt solution for 60 minutes. They were then divided into 3 groups. Group A stayed in the normal medium for 30 minutes. Groups B and C were exposed to ouabain for 30 minutes. After this period tissues in Groups A and B were removed for analysis while tissues in Group C were returned to the normal medium for 45 min to wash ouabain out. The results (Figure 5) show that ouabain caused significant ($P < 0.01$) increase in intracellular Na and decrease in intracellular K concentration. Inulin space also decreased slightly ($P < 0.05$). These effects were reversed partially on washing ouabain out. The ratio of dry to wet weight of the tissues did not differ significantly in the 3 groups and were 0.20 ± 0.01, 0.205 ± 0.02 and 0.19 ± 0.02 in groups A, B and C respectively.

Tissue ^{45}Ca content Ouabain significantly decreased the ^{45}Ca influx in taenia coli caused by increasing the potassium concentration (Figure 6).

Discussion

The initial increase in tension in the guinea-pig taenia coli caused by ouabain is associated with depolarization of the membrane along with an increase in spike frequency. Subsequently tension decreases even though membrane depolarization is maintained (Casteels, 1966). This suggests that the secondary relaxation of the taenia due to ouabain occurs by a non-electrical mechanism. Casteels (1966) suggested that the mechanism of relaxation is a depolarization block. This seems unlikely because depolarization due to high-potassium leads to a sustained increase of tension in the taenia (Imai & Takeda, 1967). Griffin *et al.* (1972) suggested that depolarization prevented the inhibitory effect of ouabain. These authors did not consider the role of absence of sodium in their depolarizing solution. The results of the present experiments suggest strongly that it is not depolarization but the possible intracellular accumulation of sodium that is responsible for the inhibitory effect of ouabain. The lack of inhibitory effect of ouabain in the absence of external sodium both in the presence or absence of hypertonicity is not an artefact of an abnormal contractile mechanism or cell volume change. Tension produced under such conditions is associated with active state and can be reduced, (more slowly than usual) by removal of extracellular calcium (unpublished observation). The same explanation may be applicable for the inhibitory effects of K-free medium (Griffin *et al.*, 1972, unpublished observations) since it is well known that absence of external potassium inhibits the Na-K pump (Skou, 1965). The intracellular accumulation of sodium which is believed to be responsible for inhibition of tension in the taenia is due to inhibition of the Na-K pump. Griffin *et al.* (1972), observed that the maximum inhibitory effect of a 5 min exposure of ileum to ouabain was seen 10 min after washing ouabain out. The simplest interpretation of this phenomenon is that the maximum accumulation of sodium in the tissue lagged behind inhibition of the Na-K pump. Gradual washout of ouabain in the above experiments could also have slowed the rate of recovery of the Na-K pump from inhibition. The changes in intracellular sodium concentration in the taenia coli correlate reasonably well with the decrease in tension produced by ouabain. The strongest evidence for an intracellular inhibitory role of sodium comes from the experiments where the effect of sodium replacement was compared with the result of substituting lithium for sodium. Sodium ions can be pumped out when the Na-K pump recovers, thus explaining the gradual recovery from inhibition. On the other hand, lithium ions which can enter the cell cannot be pumped out by the Na-K pump (Skou, 1965). Lithium can substitute for sodium in many instances (Katase & Tomita, 1972), and it is possible that the inhibitory effect on taenia is no exception. Axelsson (1961) has shown that in the taenia coli, lithium substitution for sodium abolished mechanical responses without affecting electrical activity. Perhaps a similar effect of sodium is not seen normally, because the Na-K pump prevents intracellular accumulation of sodium. The intracellular potassium concentration is also reduced by ouabain. This is unlikely to be the cause of the inhibition because elevation of extracellular potassium failed to prevent the inhibition (though the time course of inhibition was somewhat slower).

The mechanism whereby an increase in intracellular sodium inhibits contractility cannot be understood at present. It could either be at the level of the excitation-contraction process or the contractile protein. Inhibition of potassium-induced uptake of ^{45}Ca by ouabain in association with increase in intracellular Na suggest an effect on mechanisms that regulate intracellular [Ca] concentration. This effect of intracellular [Na] in the taenia coli contrasts with the increase in intracellular entry of [Ca] due to a Na-Ca exchange mechanism (Reuter, Blaustein & Haeusler, 1973) seen when intracellular [Na] is elevated in other tissues (e.g. squid axon, heart).

It is note-worthy that the inhibitory effect of cardiac glycosides is not seen in multi-unit vascular smooth muscle (Cushny, 1925; Leonard, 1957) or in cat spleen capsule (unpublished observations) although the excitatory effect is present. Thus visceral smooth muscle seems to be exceptional in this respect.

The valuable assistance of Tony Ma, D. Dahl and J. Miles is gratefully acknowledged. This work was supported by a grant from the Medical Research Council of Canada.

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(Received October 14, 1974.
Revised January 27, 1975.)

Br. J. Pharmac. (1975), 55, 117-124

FORECASTING THE ANTIMALARIAL ACTIVITIES OF ARYLAMIDINOUREAS FROM THEIR MEASURED PHYSICOCHEMICAL PROPERTIES

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1 The octanol : water partition coefficient (PC), protein binding coefficients to albumin (*Ba*) and haemoglobin (*Bh*), proton magnetic resonance (PMR) chemical shifts and pK_a of 8 arylamidinoureas have been measured.

2 There were statistically significant correlations for 7 of the 8 compounds between the predicted lipophilicity parameter π and log PC, *Ba* and *Bh*, and also between Hammett's electronic parameter σ and the PMR and pK_a observations.

3 3-Chloro,4-nitrophenylamidinourea showed a larger deviation from these correlations than any other compound. Geometric calculations based on the covalent radii and bond angles of the molecule suggested that the neighbouring chloro and nitro substituents were interacting sterically.

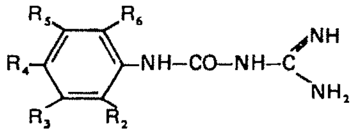
4 Molecular orbital calculations and the observed ultra-violet spectrum provided further evidence for such an interaction, and suggested that the nitro group is twisted out of the plane of the benzene ring in this molecule.

5 Such steric interactions can introduce an additional source of error when attempts are made to correlate the biological and predicted physicochemical properties of compounds.

6 Generalized parameters such as π and σ cannot be used with confidence to predict the properties of compounds in which such steric interactions occur.

Introduction

It was found by Goodford, Norrington, Richards & Walls (1973) that the antimalarial activity A of an arylamidinourea in the series with general formula:



(I)

could be forecast by the equation:

$$A = 0.28\pi + 0.86\sigma - 0.09 \qquad (1)$$

where π and σ were predicted values for two of the physicochemical properties of the compound. The equation gave adequate forecasts for the activities of 12 out of 13 new arylamidinoureas, although it had a multiple correlation coefficient of only 0.68. The possibility that a better correlation would be obtained by the use of measured properties has now been examined.

Methods

Physicochemical parameters

The effect of changing the benzenoid substituent groups R_3 to R_5 upon the lipophilicity of the arylamidinoureas was predicted with the parameter π (Leo, Hansch & Elkins, 1971). This was estimated from substituted phenols in view of the electron-donating nature of the amidinourea side-chain. Hammett's (1940) constant σ which measures the electron-withdrawing properties of the substituents was the second predicted parameter. When more than one benzenoid substituent group was present, the total change in physicochemical properties was predicted by linear addition of the individual substituent parameters.

Protein binding

(a) *Binding to albumin* All solutions were made in 0.1 M sodium phosphate buffer pH 7.4. Binding was determined after equilibrium dialysis for 18 h at 37°C of 5 ml drug solution against a sac containing 5 ml bovine serum albumin solution (fraction V. Armour Chemical Co.). Albumin concentrations up to 3×10^{-4} M were used, depending on the tightness of binding. After dialysis the concentration of unbound drug was measured spectrophotometrically or by ^{14}C counting. The dialysis sac was prepared from Visking tubing which was washed in 10^{-2} M disodium edetate (EDTA) and then soaked for 24 h in each of two changes of distilled water.

(b) *Binding to haemoglobin* Horse haemoglobin was prepared by the method of Garby, Gerber & de Verdier (1969). Solutions of drug and protein were prepared in a buffered balanced salt solution containing (mM) KCl 110, NaCl 20, MgCl 3, potassium phosphate 1, pH 7.4. Binding was determined by a modification of the ultracentrifugal procedure of Garby *et al.* (1969). Solutions of drug and haemoglobin (3×10^{-4} M) were centrifuged for 16 h at 75,000 *g* in the 6 x 15 ml swing-out rotor of the MSE Superspeed 65 Centrifuge. The upper, protein-free layer was aspirated and the concentration of free drug determined spectrophotometrically.

(c) *Expression of results* For a protein with *n* binding sites each of binding constant *K*, in equilibrium with unbound drug at concentration *D*

$$K = \frac{R}{D(n-R)}$$

where *R* is the number of drug molecules bound per molecule of protein. For the present series of compounds *n* was not known and could not be determined experimentally because of the low solubility of the test compounds. Over a range of concentrations of each drug the ratio *R/D* did not change significantly and we conclude that $n \gg R$ over the range of concentrations available to us. Results were therefore calculated as *R/D* ($\simeq nk$) which should be proportional to *K* on the not unreasonable assumption that *n* is constant for a congeneric series such as that studied in the present investigation.

For comparison with the other parameters the binding results are stated on logarithmic scales:

B_a = log (*R/D*) for albumin
B_h = log (*R/D*) for haemoglobin

Partition coefficient

Each compound was dissolved in 6 mM $NaOH/Na_2B_4O_7$ buffer at pH 9.95 and a concentration giving an absorbance of 0.2-0.8 at a suitable wavelength in the range 230-290 nm. Serial dilution measurements demonstrated the absence of phenomena which could lead to apparent deviations from Beer's Law. The absorbance A of the initial solution was determined (A initial) and 100 ml aliquots were then added to 5.0 ml octanol (Koch-Light 'Puriss') in 250 ml conical flasks, and the mixture equilibrated with gentle intermittent shaking for 1 week. The absorbance of the aqueous phase was redetermined (A final) after 30 min centrifugation at 600 *g*, and the partition coefficient (PC) calculated as:

$$PC = \frac{\text{A initial} - \text{A final}}{\text{A final}} \times 20 \qquad (2)$$

Proton magnetic resonance

The proton magnetic resonance spectrum for each compound was determined by Mr A.G. Ferrige at 100 MHz on a Varian HA-100 using a probe temperature of 27°C. Hexadeuterodimethylsulphoxide was used as the solvent and a concentration of approximately 100 mg/ml was employed. A capillary containing 10% hexamethyldisiloxane dissolved in carbon tetrachloride was used to establish a field-frequency lock. Chemical shifts of the aromatic protons were measured in parts/10^6 from the lock signal.

Apparent pK_a

Each compound was dissolved at a concentration of approximately 1 mM in 20 mM HCl, and 0.1 or 0.2 ml pipetted into 3 ml phosphate buffer in a 1 cm path-length silicon cuvette at room temperature. Ultra-violet absorption spectra were recorded on a Unicam SP800 spectrophotometer for a range of 7 or 8 buffers between pH 6.2 and 7.5 at ionic strength of 0.1. The pH of each solution was verified with Radiometer Blood pH equipment. Values of absorbance A^λ were measured from the recorded spectra for several different wavelengths λ.

Values of pK_a $A^\lambda{}_{BH^+}$ and $A^\lambda{}_B$ were fitted for each wavelength λ from equation (3).

$$pH - pK_a = \log_{10}\left(\frac{A^\lambda - A^\lambda{}_{BH^+}}{A^\lambda{}_B - A^\lambda}\right) \qquad (3)$$

using an iterative calculation. This method avoids the necessity of measuring the absorbance of the base in dilute acid and dilute alkali, when second order medium effects may perturb the spectrum. Each final pK_a value is derived from observations at four different wavelengths.

Results

Protein binding

Observations were made on a group of eight arylamidinoureas (Table 1) chosen to have ranges of π and σ as wide and uncorrelated as practicable. These were the same compounds as were used by Cranfield, Goodford, Norrington, Richards, Sheppey & Williams (1974). It was found that haemoglobin binding B_h was related to π according to the equation:

$$B_h = 0.36\pi + 2.78 \qquad (5)$$

$$t = 4.35; \quad F = 18.9;$$
$$d.f. = 6; \quad 0.005 > P; \quad M.C.C. = 0.87;$$

which shows that lipophilic arylamidinoureas tended to bind more strongly than hydrophilic compounds.

By contrast, there was a less satisfactory relationship between albumin binding B_a and π:

$$B_a = 0.65\pi + 3.46 \qquad (6)$$

$$t = 2.53; \quad F = 6.39; \quad d.f. = 6;$$
$$0.05 > P > 0.02; \quad M.C.C. = 0.72$$

and when the results were plotted it was found that the poorer statistics were particularly due to the deviation of the 3-chloro,4-nitroarylamidinourea from the regression line. The residual for this compound was more than double that of any other, and the regression for the remaining seven arylamidinoureas was more acceptable:

$$B_a = 0.45\pi + 3.50 \qquad (7)$$

$$t = 4.14, \quad F = 17.14; \quad d.f. = 5;$$
$$0.01 > P > 0.005; \quad M.C.C. = 0.88$$

Equation (7) is plotted in Figure 1a, which demonstrates the deviation of the 3-chloro, 4-nitro compound from the regression for the other seven, and it was this finding which first suggested the possibility that π might be an inadequate prediction of lipophilicity in this series of compounds.

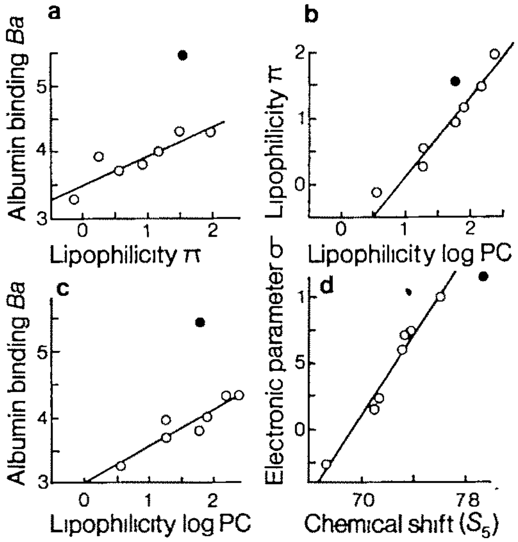


Figure 1 The relationships between predicted physicochemical properties (π and σ) and those observed (B_a, log PC, S_5). Logarithmic units. Each circle represents one of the eight compounds. The black circle represents the anomalous 3-chloro, 4-nitro compound, and the linear regressions are least-squares fits to the other seven points. See text.

Partition coefficient

The partition coefficients (PC) for the same eight compounds were determined at high pH in order to suppress ionization, and were related to π according to the equation

$$\pi = 1.15 \log PC - 0.92 \qquad (8)$$

$$t = 7.38; \quad F = 54.52, \quad d.f. = 6,$$
$$0.001 > P; \quad M.C.C. = 0.95$$

Table 1 The benzenoid substituent groups, predicted parameters and measured properties of the eight arylamidinoureas

Substituent groups		*Predicted parameters*		*Observed protein binding*		*Observed physicochemical properties*		
R_3	R_4	π	σ	B_h	B_a	log PC	S_5	pK_a
meta	para	*lipophilicity*	*electronic*	*Haemoglobin*	*Albumin*	*log partition coefficient*	*Proton 5 chemical shift*	*Acidity*
H	OCH_3	−0.12	−0.27	2,53	3.26	0.56	6.71	7.73
H	Cl	0.93	0.23	2.98	3.79	1.78	7.14	7.36
NO_2	H	0.54	0.71	3.11	3.68	1.28	7.34	7.23
Cl	CN	1.18	1.00	3.32	4.01	1.90	7.62	6.95
Cl	Cl	1.97	0.60	3.51	4.31	2.39	7.32	7.21
Cl	NO_2	1.54	1.15	3.34	5.43	1.79	7.94	7.17
OCH_3	CN	0.26	0.74	3.09	3.94	1.28	7.39	6.96
CH_3	Cl	1.49	0.16	3.22	4.31	2.19	7.10	7.58

which was statistically acceptable with a slope that did not differ significantly from unity. Once again the 3-chloro,4-nitroarylamidinourea had the largest residual error (Figure 1b), and when it was omitted the equation became

$$\pi = 1.12 \log\mathrm{PC} - 0.93 \qquad (9)$$

$t = 9.57$; $F = 91.6$; $d.f. = 5$;

$0.001 > P$, $M.C.C. = 0.97$

However, the π value for this compound deviated from the π: log PC regression (equation (9) in the wrong direction to explain the discrepant point in Figure 1a, and the 3-chloro,4-nitroarylamino-urea therefore showed an even larger residual error when albumin binding B_a was regressed directly against log PC for all eight compounds:

$$B_a = 0.63 \log\mathrm{PC} + 3.05 \qquad (10)$$

$t = 1.74$; $F = 3.04$; $d.f. = 6$;

$0.2 > P > 0.1$; $M.C.C. = 0.58$

When the individual points are examined (Figure 1c) the anomalous behaviour of this one compound is still more clearly displayed, particularly in contrast to the satisfactory regression of B_a against log PC for the remaining seven arylamidinoureas:

$$B_a = 0.55 \log\mathrm{PC} + 3.01 \qquad (11)$$

$t = 5.79$; $F = 33.52$; $d.f. = 5$;

$0.005 > P$; $M.C.C. = 0.93$

Proton chemical shift

In view of the anomalies of the 3-chloro, 4-nitro compound in the previous observations, it was of interest to establish whether σ provided a reliable prediction of the electron-withdrawing properties of these two substituent groups in adjacent positions on the arylamidinourea benzenoid ring. In the absence of shift measurements for the C-1 carbon atom, the proton bonded to the nitrogen atom at position 1 of the ring would best reflect changes in substituent σ values, but for the complications arising from proton exchange. However, substituents at position 3 perturb the electrons at positions 1 and 5 equally via a field effect. Moreover, substituents at position 4 perturb the electrons at positions 1 and 5 by an essentially similar resonance effect. It was therefore not unreasonable to study the chemical shift S_5 of proton 5, and this was found to have a close correlation with σ:

$$\sigma = 1.25\, S_5 - 8.62 \qquad (12)$$

$t = 9.74$; $F = 95.01$; $d.f. = 6$;

$0.001 > P$; $M.C.C. = 0.97$

As previously, the same compound had the largest residual error (Figure 1d) and when it was omitted the regression equation became:

$$\sigma = 1.49\, S_5 - 10.32 \qquad (13)$$

$t = 12.92$; $F = 167.04$; $d.f. = 5$;

$0.001 > P$; $M.C.C. = 0.99$

which is plotted on the figure.

pK$_a$

Although the chemical shift S_5 correlated well with σ it is not possible to justify rigorously the arguments in the previous section in favour of studying position 5. An attempt was therefore made to study the effect of the benzenoid substituent groups upon the main side-chain directly. Since the amidinourea moiety is partly ionized in the physiological range, it seemed possible that pK_a observations might not only show the relevant electron- distribution in the side-chain, but might also be directly related to the biological activities of the compounds. However, the relationship between σ and pK_a:

$$\sigma = -1.51\, \mathrm{pK_a} + 11.52 \qquad (14)$$

$t = 4.56$; $F = 20.83$; $d.f. = 6$,

$0.005 > P > 0.001$; $M.C.C. = 0.88$

was not as close as the relationship between σ and S_5. The 3-chloro,4-nitro compound once again had the largest residual error, and the t-value increased when it was omitted:

$$\sigma = -1.41\, \mathrm{pK_a} + 10.69 \qquad (15)$$

$t = 6.92$; $F = 47.88$; $d.f. = 5$;

$0.001 > P$; $M.C.C. = 0.95$

The anomalies of this one compound therefore account for the poorer results to some extent. However, a further factor may be that the observed ionization occurs at the terminal amidine group. This is isolated by the rest of the side-chain from the resonance effects of the benzenoid substituents, and hence the pK_a observations may only be indirectly related to sigma.

Steric interactions

It seemed possible that some of the anomalies of the 3-chloro, 4-nitro compound might be due to steric interactions between the chlorine and its adjacent oxygen atom. The distance between their atomic centres was therefore calculated, taking reasonable values (Table 2) for covalent radii and bond angles, and was found to be 2.5 Å assuming that all the atoms were in the plane of the benzene

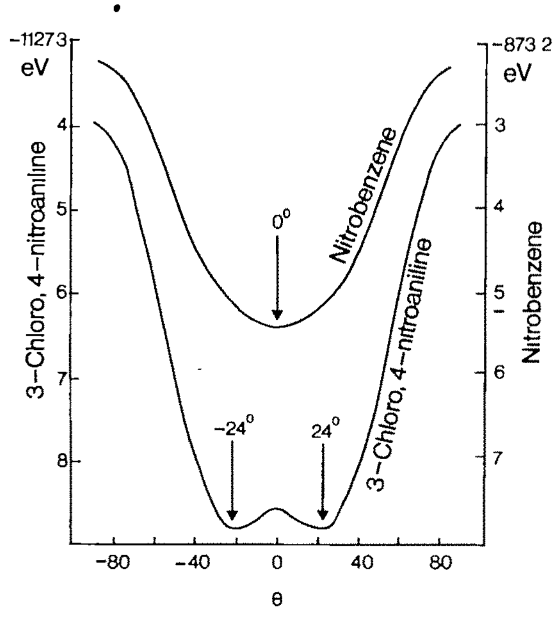


Figure 2 Results of molecular orbital calculations by the extended Hückel Treatment on 3-chloro, 4-nitroaniline and nitrobenzene. The nitrogen and chlorine atoms were constrained to lie in the plane of the benzene ring. Abscissae, the angle of rotation θ between this plane and the plane of the $-NO_2$ substituent group. Ordinates, the total energy calculated in eV. For nitro benzene there is a single minimum when the planes are parallel ($\theta = 0°$), but for 3-chloro, 4-nitroaniline there is a potential energy barrier at coplanarity and double minima at $\theta = \pm 24°$. See text.

ring. It is generally accepted that steric interference may occur in such situations if atoms approach nearer than twice the sum of their covalent radii (Braude & Sondheimer, 1955), which are approximately 0.99 Å and 0.55 Å for chlorine and doubly bonded oxygen respectively. Hence the interference spheres would touch at a centre-to-centre distance of 3.08 Å, and the calculated value of 2.5 Å indicates an unacceptable proximity unless the nitro group were rotated out of the plane of the benzene ring, and/or the chlorine atom displaced.

In a further attempt to predict this interaction, molecular orbital calculations were performed on nitrobenzene and 3-chloro,4-nitroaniline according to the extended Huckel treatment (Hoffman, 1963) using the bond lengths and angles shown in Table 2. The hydrogen, chlorine and nitrogen atoms were constrained to stay in the plane of the benzene ring, and the total binding energy E was calculated for each molecule as a function of the angle θ between the plane of the nitro group and the plane of the benzene ring. This energy was calculated for 10° increments of θ, and a single minimum at $\theta = 0°$ was found for nitrobenzene (Figure 2) whereas for 3-chloro, 4-nitroaniline a potential energy barrier was found for this planar conformation above a double minimum at ± 24°.

Such a calculation can only be approximate, because it is subject to the limitations of the extended Hückel method and the choice of atomic parameters. Nevertheless, it again shows that there may be a steric interaction between neighbouring chloro and nitro benzenoid substituents which can be relieved if the nitro group rotates out of the plane of the benzene ring.

For benzenoid substituents which have π electrons or lone pairs overlapping the π orbitals of the benzene ring, it is well established that steric perturbations can lead to changes in mesomeric interaction and absorption intensity of the ultra-violet absorption band usually seen in the 260-280 nm region (Braude & Sondheimer, 1955, Baddeley, Smith & Vickars, 1956; Frolen & Goodman, 1961). In the present case a single chloro substituent has little effect on the ultra-violet absorption of benzene (Table 3), but a single nitro group enhances the absorption intensity greatly. When both are present and unhindered as in the *para* isomer 1-chloro, 4-nitro benzene, the substituents interact and actually give a still higher intensity. However, positioning the nitro substituent *ortho* to the chloro in 1-chloro, 2-nitrobenzene has the reverse effect, and this is again compatible with a rotation of the nitro group about the C–N bond axis. There may also be a simultaneous bending of the Cl atom out of the plane of the benzene ring (Forbes, 1960), but on either interpretation one must conclude that the adjacent benzenoid chloro and nitro groups interact sterically.

Table 2 The covalent bond radii and angles used to calculate the chlorine-oxygen distance in 3-chloro, 4-nitro phenylamidinourea and 3-chloro, 4-nitroaniline

For benzene:	CC = 1.40 Å	CCl = 1.70 Å	CCC = 120°
For nitro:	CN = 1.48 Å	NO = 1.21 Å	ONO = 124°
For amino	CN = 1.43 Å	NH = 1.01 Å	HNH = 120°

Discussion

The protein binding studies were initiated in order to ascertain whether the antimalarial activity of arylamidinoureas was associated with a selective binding to haemoglobin. This might either facilitate the uptake of drug by the parasite, or make the haemoglobin less available as a source of nutrient. However, the results lend no support to either hypothesis, as the ratio *Bh/Ba* for arylamidinoureas is similar to that of other small aromatic molecules (Hansch, Kiehs & Lawrence, 1965, Kiehs, Hansch & Moore, 1966). Any correlation between antimalarial activity and protein binding is therefore probably a reflection of their mutual correlation with lipophilicity.

The present results show little evidence against the use of π to predict lipophilicity and σ to predict electron-withdrawal in seven out of the eight arylamidinoureas studied. However, the 3-chloro,4-nitrophenylamidinourea shows a number of anomalies, and it is reasonable to interpret these in terms of the intramolecular steric hindrance in this compound. Twisting the nitro group out of the plane of the benzene ring disrupts the mesomeric interaction between the two, and thereby invalidates σ calculated from tabulated values in the literature. The 'corrected' σ is lower and is therefore more compatible with the compound's observed pK_a. Moreover, Fujita, Iwasa & Hansch (1964) have shown that the π value for any substituent is related to its σ value. Hence the 'corrected' σ for 3-chloro, 4-nitro phenylamidinourea leads to a 'corrected' π value which similarly reduces the discrepancy between log PC and π for this compound.

If the 4-nitro group is forced out of the plane of the benzene ring by the influence of the neighbouring 3-chloro substituent, it will itself have an anomalous effect upon the other substituent positions in the benzene ring. Hence it is not surprising that this compound has the largest residual error in the $\sigma : S_5$ correlation, and the proton chemical shift may in fact be a useful experimental probe for investigating such steric effects.

Table 3 Ultra-violet spectral characteristics of substituted benzenes. The depression of extinction coefficient from 8,900 for nitrobenzene to 4,000 in 1-chloro,2-nitrobenzene demonstrates a steric interaction between these *ortho* substituent groups.

Compound	λ_{max}	ϵ
Chlorobenzene	253	400
Nitrobenzene	253	8,900
1-Chloro, 4-nitro benzene	265	12,000
1-Chloro, 2-nitro benzene	244	4,000

Cranfield *et al.* (1974) observed the antimalarial activities of the present eight compounds in three test systems: *Plasmodium vinckei* in mice *in vivo*; *P. knowlesi* in monkey erythrocytes *in vitro*; and *P. berghei* in rat erythrocytes *in vitro*. They then calculated regression equations against π and σ for each activity, and used their equations to forecast the activities of further compounds. However, the present findings throw doubt on any equations which rely on predicted values of π and σ for the 3-chloro,4-nitrophenylamidinourea, and we have therefore tested alternative strategies in order to get over the difficulties raised by such a compound.

The findings of Cranfield *et al.* (1974) are summarized in Table 4 (method 1) for comparison with three alternative methods of calculation. With each of these alternatives it is accepted that the predicted π and σ for the 3-chloro,4-nitro compound are anomalous, but different methods are adopted for dealing with the anomalies. In method 2 (Table 4) the anomalous compound is ignored altogether, and the regressions calculated for the other seven. In method 3 the regressions are calculated for all eight compounds using the observed physicochemical measurements log PC and S_5. Finally, in method 4 'corrected' values of π and σ are calculated for the one compound from its measured log PC and S_5 values and from equations (9) and (13). These 'corrected' values are then used with the predicted π and σ parameters for the other seven compounds in order to derive regression equations.

No one method is consistently superior to the other three, when the conventional statistics (variance ratio *F* and multiple correlation coefficient *R*) of the equations are compared. For example with *P. vinckei* in mice the first method gives an *F* ratio of 6.4 with 5 and 2 degrees of freedom which is significant at the 5% probability level, while the second method only gives 3.4 with 4 and 2 d.f. which is not even significant at 10%. However, an opposite trend is observed when the same methods are compared for *P. knowlesi* where the *F* statistic (21.9) for method 2 is significant at the 1% probability level while method 1 fails to satisfy the same criterion. In fact neither method 1 nor method 2 demonstrates a clear superiority, and one must conclude after further study of the *F* and *R* values that these statistics do not afford any reliable guide for choosing between methods on the basis of the present observations. However, it will be observed in Table 4 that the coefficient of the lipophilicity term is always positive for the *P. vinckei* observations, and that it is always much less than the coefficient of the electronic term

Table 4 A comparison of regression equations derived by four different methods (see text)

		Method of deriving equation															
		Method 1				*Method 2*				*Method 3*				*Method 4*			
Parasite	*Host*	*8 Compounds predicted π and σ*				*7 Compounds predicted π and σ*				*8 Compounds observed log PC and S_5*				*8 Compounds, 7 predicted 1 from observations*			
		F	*R*	π	σ	*F*	*R*	π	σ	*F*	*R*	*log PC*	S_5	*F*	*R*	π	σ
P. vinckei	Mice *in vivo*	6.4	0.85	0.35	0.78	3.4	0.79	0.34	0.73	6.5	0.85	0.28	1.18	6.6	0.85	0.34	0.75
P. knowlesi	Monkey *in vitro*	11.9	0.91	0.88	0.21	21.9	0.96	0.93	0.47	38.3	0.97	1.16	0.21	20.2	0.94	0.96	0.25
P. berghei	Rat *in vitro*	11.1	0.90	0.53	−0.54	9.2	0.91	0.54	−0.51	11.0	0.90	0.66	−0.61	8.7	0.88	0.52	−0.35

Under each method is given the variance ratio *F*, the multiple correlation coefficient *R*, the coefficient of the lipophilicity term and the coefficient of the electronic term in the equation. The first method is that of Cranfield *et al.* (1974).

(row 1). This precedence is reversed for *P. knowlesi* where the electronic term plays a minor role, while for *P. berghei* its sign is consistently reversed. Hence each test system yields a characteristic type of equation, irrespective of the method of calculation.

The consistency of the equations is further demonstrated when they are used to forecast the antimalarial activities of more arylamidinoureas. Cranfield *et al.* (1974) studied a total of 44 forecasts, all of which were made on fresh compounds that had not been used to derive the regression equations. They assessed the forecasting ability of an equation by comparing the ranking order of forecast potency with the ranking order of measured antimalarial activity of the compounds, and this same method has been used to test the equations from methods 2 and 4 for comparison with method 1 which was that used by Cranfield *et al.* themselves. Method 3 cannot be used for forecasting at all, since the equations use the measured properties log PC and S_5 which would not be known for any novel compound until it had actually been synthesized.

The ranking order of 9 forecasts for *P. knowlesi* activity was the same for methods 1, 2 and 4, and there was only one small change in the 9 forecasts for *P. berghei* (ranks 4 and 5 were reversed for method 4). All 26 forecasts for *P. vinckei* were the same for all methods, except that ranks 3 and 4 were reversed for method 1, and there is very little to choose between the forecasting properties of the equations derived by methods 1, 2 and 4. However, it would be unwise to generalize from these findings, and in future investigation it would be much more straightforward to use compounds for which steric interactions do not occur. The probability of bulky groups interacting can be quickly assessed by geometric calculations using published covalent atomic radii, and compounds can be selected for study where this pitfall will be avoided. If this is done at the early stages of a physicochemical-activity relationships investigation, when the regression equations are first being calculated, it should be possible to retain all the advantages of the approach and eliminate one potential source of error.

We are grateful to Mr O. Fanimo, Mr A.G. Ferrige, Mr M.J. Norman, Miss J. Peacock, Dr W.H.G. Richards, Mr G.C. Sheppey, Mrs N. Trist and Dr L.P. Walls for much valued help and discussion in the course of this work.

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(Received February 3, 1975.
Revised June 3, 1975.)

Br. J. Pharmac. (1975), 55, 125-131

DESENSITIZATION IN THE INNERVATED AND IN THE CHRONICALLY DENERVATED SOLEUS MUSCLE OF THE MOUSE

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1 A study was made of desensitization in chronically denervated and in normally innervated mouse soleus muscles.

2 Very high concentrations of acetylcholine produced small contractions of the innervated muscles; these were reduced in size when the addition was repeated 1 min after wash-out.

3 Desensitization in innervated muscles was receptor specific: contractions in response to KCl and caffeine were not reduced following acetylcholine, nor did KCl produce desensitization.

4 In chronically denervated muscles non-specific desensitization was observed if acetylcholine was added in the presence of low concentrations of acetylcholine or carbachol. Contractions to KCl but not to caffeine were reduced. KCl produced a similar kind of desensitization.

5 After washing out moderate or supramaximal concentrations from the chronically denervated muscle no desensitization was observed. However, 1 min after washout of very high concentrations the muscle was non-specifically desensitized.

6 It is concluded that relatively high concentrations of acetylcholine are required to produce specific desensitization in the soleus muscle. Chronically denervated muscles, being supersensitive, show non-specific loss of sensitivity with concentrations of acetylcholine too low to produce specific desensitization.

Introduction

Katz & Thesleff (1957) observed a reduction in the depolarization of frog muscle endplates produced by acetylcholine when iontophoretically applied during a longer conditioning pulse of acetylcholine. They postulated that this was due to a change in the receptor properties on prolonged activation and proposed a cyclic model where the drug-receptor complex changes to a desensitized form which reverts only slowly to the active form. Using the chick biventer, Rang & Ritter (1970) provided evidence that desensitization involves receptor inactivation and mathematically analysed various possible mechanisms. They found that the rate constant of recovery from desensitization was the same for all agonists and this, in conjunction with their other data, was held to favour the cyclic model proposed by Katz & Thesleff (1957). Comparable experiments have not been performed in denervated muscle where receptors are formed *de novo* although desensitization is known to occur (Axelsson & Thesleff, 1959; Miledi, 1960a). After chronic denervation acetylcholine receptors can be demonstrated outside the endplate zone to which receptors in innervated fibres are confined (Axelsson & Thesleff, 1959). We have examined the desensitization process at these receptors in the chronically denervated mouse soleus muscle.

Methods

Male mice weighing 20-30 g were anaesthetized with ether. The left sciatic nerve was aseptically sectioned in the popliteal space and about 1 cm removed. The wound was closed and the animals allowed to regain consciousness. After 7 to 12 days the animals were killed by a blow on the head and the soleus muscles were dissected from both legs. The denervated muscles weighed about 12% less than those from non-operated legs.

The muscles were suspended in 5 ml baths containing Krebs-Henseleit solution of the following composition (mM): Na^+ 143, K^+ 5.8, Mg^{2+} 1.2, Ca^{2+} 2.5, $H_2PO_4^-$ 1.2, HCO_3^- 25, Cl^- 128, SO_4^{2-} 1.2, glucose 11.1, which was maintained at 37°C and bubbled with 5% CO_2 in O_2. Tension was measured with Grass FTO3 C or Nihon Kohden 58-IT isometric transducers and

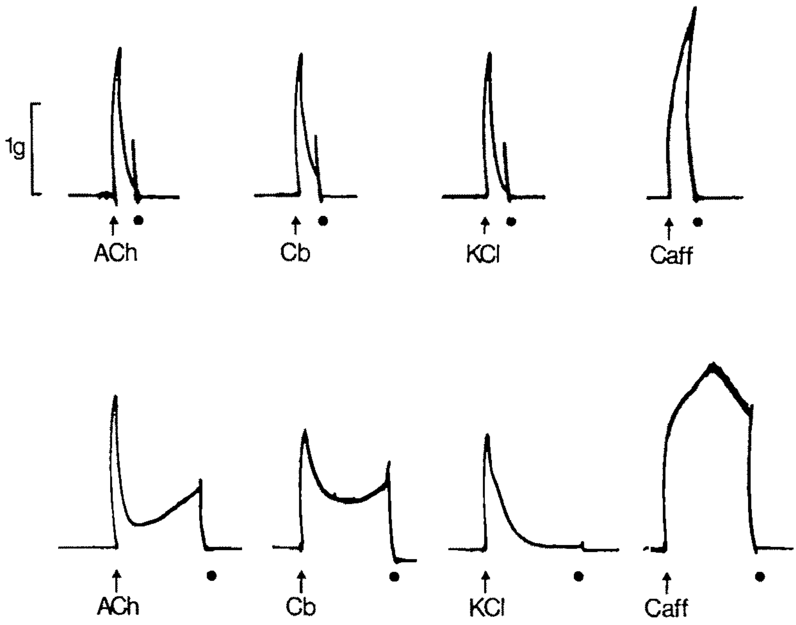

Figure 1 Contractions of the chronically denervated mouse soleus muscle obtained by the addition of acetylcholine 5.5×10^{-6} M (ACh), carbachol 5.5×10^{-6} M (Cb), potassium chloride 100 mM (KCl) or caffeine 26 mM (Caff). The drugs were washed out (●) after 1 min (top row) or after 4 min (bottom row).

recorded on a Grass 7B polygraph. The resting tension was adjusted to 1 gram.

The drugs used were acetylcholine chloride (Sigma), caffeine citrate (Evans), carbachol chloride (BDH). The concentrations quoted in the text are the final bath concentrations.

Results

Chronically denervated

Acetylcholine in concentrations greater than 5.5×10^{-7} M produced contractions of the denervated mouse soleus. The contraction reached a maximum in about 6 s and tension then returned towards the baseline (Figure 1). If the contact time was 4 min, a second phase of the contraction was normally seen (Figure 1). In some preparations the second phase became more prominent during the course of the experiment, although in 4 out of 15 experiments it was absent. In two preparations the second phase was greater than the first. With higher concentrations of acetylcholine, the second phase was found to develop more rapidly. A wash contraction was normally produced after rinsing out acetylcholine (Figure 1).

Contractions in response to carbachol (in concentrations greater than 5.5×10^{-7} M) and potassium chloride (KCl) (10-150 mM) were similar in form to those in response to acetylcholine, except that KCl did not produce a second phase on prolonged exposure (Figure 1). Contractions to caffeine (2.6×10^{-3}—2.6×10^{-2} M) developed more slowly, but the tension was maintained during 4 min contact time (Figure 1).

Dose-response curves were constructed for acetylcholine, carbachol, caffeine and KCl (Figure 2). The acetylcholine and carbachol curves were parallel, acetylcholine being 2.7 times more potent than carbachol. The 50% maximum contraction (determined by inspection of the graph) required 7.6×10^{-7} M acetylcholine. The slopes of the caffeine and KCl dose-response curves were greater than the acetylcholine or carbachol curves. The maximum contraction produced by caffeine was significantly greater than that produced by acetylcholine or carbachol ($P < 0.01$).

Desensitization, as described by Rang & Ritter (1970), could not be obtained in the chronically denervated mouse soleus muscle. No effect at all was observed 1 or 3 min after washout of submaximal (5.5×10^{-6}—5.5×10^{-5} M) or even supramaximal (5.5×10^{-5}—2.8×10^{-4} M) con-

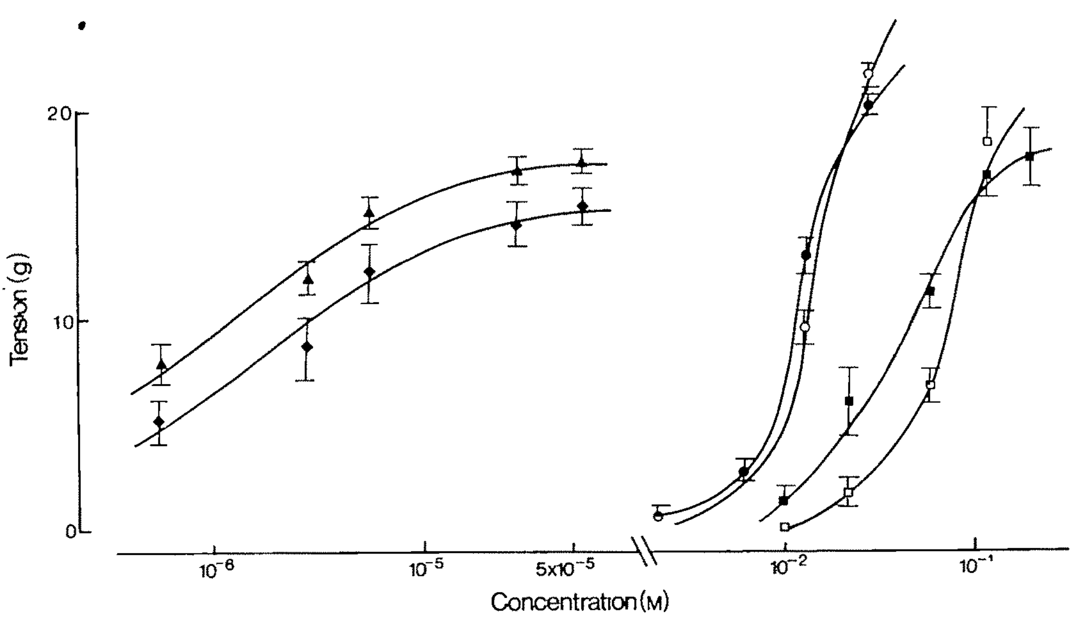

Figure 2 Concentration-response curves obtained using the chronically denervated (closed symbols) and the innervated (open symbols) mouse soleus muscles. Contractions were produced by acetylcholine (▲), carbachol (◆), caffeine (●) or potassium chloride (■) in the denervated preparations and by caffeine (○) or potassium (□) in innervated preparations. Each point is the mean of 6 observations, vertical bars show s.e. mean.

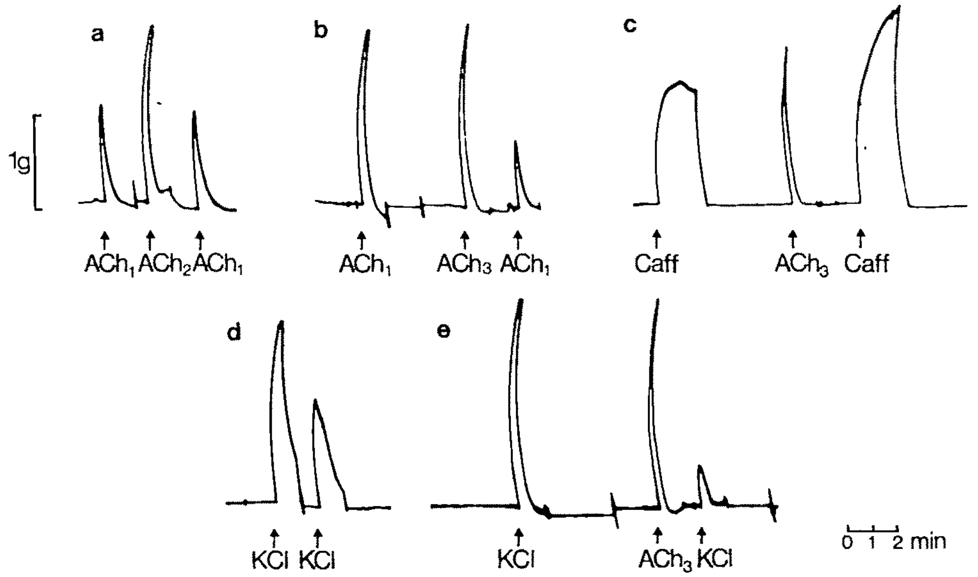

Figure 3 Contractions of the chronically denervated mouse soleus muscle. The drugs were added at the points indicated by the arrows and in each case were washed out after 1 minute. (a) A control response (ACh$_1$) was obtained with acetylcholine (5.5×10^{-6} M). After a 10 min rest (during which the recording was stopped), acetylcholine 2.8×10^{-4} M (ACh$_2$) was added and 1 min after washing out this high dose another test addition of acetylcholine (5.5×10^{-6} M) was made (ACh$_1$). (b) The first response is a control, obtained with acetylcholine 5.5×10^{-6} M (ACh$_1$). The preparation was rested and then a very high dose of acetylcholine given (ACh$_3$), 5.5×10^{-4} M). One minute after washout, the test dose was repeated (ACh$_1$). (c) Caffeine, 1.3×10^{-2} M (Caff) was given first as a control response and then repeated 1 min after washing out acetylcholine, 5.5×10^{-4} M (ACh$_3$). (d) Some reduction was seen in the response to a second dose of KCl (100 mM) when applied 1 min after washing out the first dose. (e) The KCl test response (KCl, 100 mM) gave a reduced contraction when added 1 min after washing out acetylcholine (ACh$_3$, 5.5×10^{-4} M).

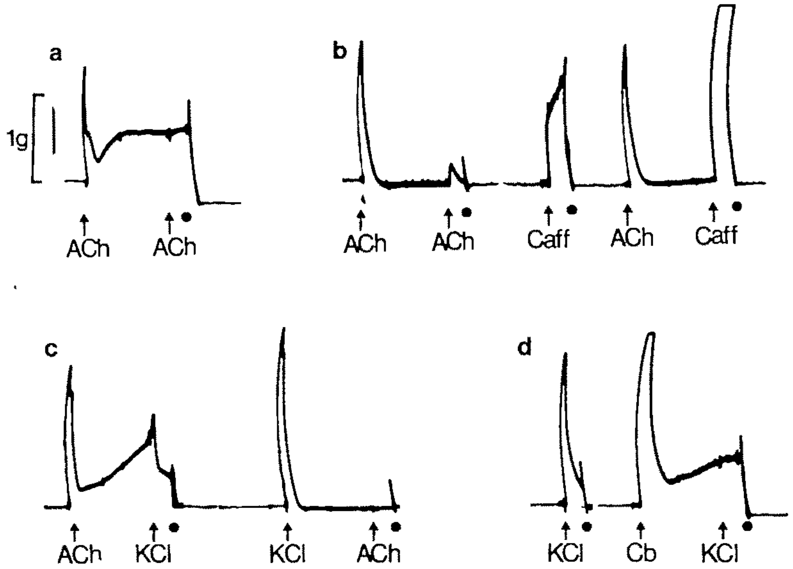

Figure 4 Contractions of four different chronically denervated mouse soleus muscles Drugs were added at the arrows. At the dots, the preparations were washed and rested for 10 minutes. (a) Acetylcholine 5 5 x 10^{-6} M caused a contraction (ACh) An identical addition repeated 4 min after the first and during the second phase of the contraction produced no effect (b) In this preparation the contraction produced by acetylcholine (ACh) (5.5 x 10^{-6} M) had no second phase. A second addition made 4 min after the first produced a small contraction only. A control response to caffeine, 13 mM (Caff) was obtained and this was augmented 4 min after the addition of acetylcholine (5.5 x 10^{-6} M). (c) Potassium chloride, 100 mM (KCl) was added during the second phase of the acetylcholine (ACh, 5.5 x 10^{-6} M) contraction in this preparation. It produced a biphasic response consisting of a smaller contraction than KCl added alone and this was immediately followed by relaxation. Acetylcholine (5.5 x 10^{-6} M) was inactive when added in the presence of KCl 100 mM. (d) In this preparation, KCl (50 mM) caused no effect when added during the second phase of the contraction produced by carbachol (Cb), (5.5 x 10^{-6} M). A similar result could also be obtained when KCl was added during the second phase of acetylcholine contractions.

centrations. Figure 3a demonstrates this lack of desensitization 1 min after washing out a conditioning concentration of acetylcholine (2.8 x 10^{-4} M) when the response to a test addition of acetylcholine (5.5 x 10^{-6} M) was unaltered. With very high concentrations of acetylcholine or carbachol (5.5 x 10^{-4} M and in some cases also with 2.8 x 10^{-4} M) desensitization was observed but was found to be non-specific. One minute after washout of very high concentrations of acetylcholine or carbachol, test responses to acetylcholine or carbachol were reduced (Figure 3b) as were responses to KCl, 100 mM (Figure 3e). This desensitization at very high conditioning concentrations of acetylcholine or carbachol was therefore not specifically related to a single receptor type. Nevertheless, caffeine contractions were not reduced by very high conditioning concentrations of acetylcholine or carbachol (Figure 3c). Further evidence that this desensitization was non-specific was obtained from the observation that high concentrations of KCl also reduced the size of test responses to KCl (Figure 3d), acetylcholine and carbachol, but not to caffeine.

In another series of experiments, conditioning concentrations of acetylcholine (5.5 x 10^{-6} M) or carbachol (5.5 x 10^{-6} M) were added and, after 4 min without washing, a test dose was administered. Test responses to acetylcholine (5.5 x 10^{-6} M), carbachol (5.5 x 10^{-6} M) and KCl (50 mM) were reduced, indicating non-specific desensitization (Figure 4). Test additions of acetylcholine or carbachol were ineffective when superimposed on the second phase of the acetylcholine or carbachol contraction (Figure 4a). However, when no second phase was present the test addition did cause a contraction, though it was much reduced in size (Figure 4b). Test additions of KCl (50 mM) added in the presence of either acetylcholine or carbachol (5.5 x 10^{-6} M) in some experiments had no effect (Figure 4d) and

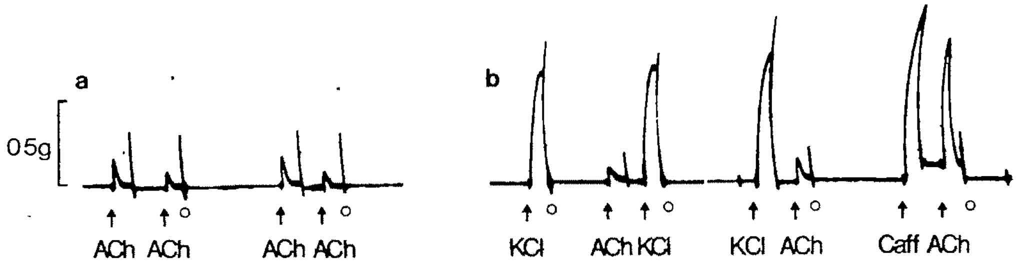

Figure 5 Contractions of the normally innervated mouse soleus preparation. Drugs were added at the arrows and washed out after 1 minute. The preparation was rested for 10 min at the points marked thus: o. (a) Acetylcholine 5.5×10^{-3} M caused a small contraction (ACh). When repeated 1 min after washing out, the contraction was reduced. This sequence was repeated. (b) A control response to potassium chloride, 50 mM (KCl) was obtained. The size of the KCl contraction was not reduced when added 1 min after washing out acetylcholine, 5.5×10^{-2} M (ACh). Also acetylcholine (5.5×10^{-2} M) gave a contraction of control size when added 1 min after washing out potassium (50 mM). However, added 1 min after rinsing out caffeine (13 mM), the response to acetylcholine, 5.5×10^{-2} M (ACh) was considerably augmented.

in others produced a small rise in tension followed by inhibition of the second phase of contraction (Figure 4c). Contractions in response to caffeine (1.3×10^{-2} M), on the other hand, were augmented when caffeine was added in the continuing presence of acetylcholine (5.5×10^{-6} M) (Figure 4b). A similar kind of non-specific desensitization was produced by KCl (50 mM), which reduced responses to acetylcholine (Figure 4c) to carbachol or to KCl, but not to caffeine.

Innervated

In innervated mouse soleus muscles, taken either from the contralateral (non-operated) leg or from intact animals, caffeine and KCl produced contractions that were similar in form to those obtained in denervated preparations, except that the KCl contractions were slightly better sustained. The dose-response curves produced by caffeine and KCl were similar in denervated and in innervated muscles (Figure 2).

Acetylcholine or carbachol in concentrations greater than 5.5×10^{-4} M usually gave small contractions although these were rather variable in size even in a single preparation. In 8 preparations the mean contraction produced by 5.5×10^{-3} M acetylcholine was 0.09 ± 0.01 g and by 5.5×10^{-2} M acetylcholine was 0.13 ± 0.02 gram. When a second addition of acetylcholine or carbachol was made 1 min after washout of a control addition of either agonist, then the response was reduced or almost abolished (Figure 5). The mean control contraction produced by 5.5×10^{-2} M acetylcholine in 10 preparations was 0.14 ± 0.01 g and on repetition 1 min after washing was 0.01 ± 0.01 gram. On the other hand, contractions to caffeine and KCl were of control size when additions were made 1 min after washing out acetylcholine. Contractions to acetylcholine were of normal size when added after washout of KCl (50 mM) and were augmented after washout of caffeine (1.3×10^{-2} M).

Discussion

In innervated preparations, desensitization was observed when test additions of acetylcholine or carbachol were made 1 min after washout of a previous dose. Since the KCl contractions were normal at this time, this desensitization appears to be a property of the receptors themselves, and resembles the receptor specific desensitization observed at the frog motor endplate (Katz & Thesleff, 1957; Nastuk, Manthey & Gissen, 1966) in the chick biventer cervicis muscle and leech dorsal muscle (Rang & Ritter, 1970) and cultured skeletal muscle (Harvey & Dryden, 1974a). This conclusion is supported by the observation that following a contraction to KCl, the sensitivity to acetylcholine was not reduced, and therefore this type of desensitization does not follow the action of all agonists. Also, the sensitivity to acetylcholine was not reduced following the action of caffeine and in fact was found to be augmented. This is probably the result of caffeine reducing the rate of calcium re-uptake by the sarcoplasmic reticulum (Weber & Herz, 1968).

In contrast to these results, a similar procedure applied to the chronically denervated mouse soleus muscle produced no desensitization at all when moderate to large doses were used. However, desensitization was observed following washout of very high concentrations of carbachol or acetylcholine ($2.8–5.5 \times 10^{-4}$ M) and this appeared to be different in type from that observed in the innervated preparations. Test responses to KCl were reduced as well as those to acetylcholine and carbachol, and moreover it was found that KCl could itself cause desensitization. This desensitization was therefore non-specific in the sense that it was not limited to drugs acting at the acetylcholine receptor. Thus the desensitization demonstrated here in the denervated soleus muscle of the mouse differs from that studied by Freeman & Turner (1972), who demonstrated, in the denervated rat diaphragm, cross desensitization among some nicotinic agonists but not between acetylcholine and carbachol. Following pretreatment with acetylcholine, the muscle was not desensitized to caffeine and therefore it is likely that the non-specific desensitization process involves a change in the electrical or permeability characteristics of the plasma membrane as caffeine is considered to act intracellularly at the level of the sarcoplasmic reticulum (Ogawa, 1970).

In chronically denervated muscles, non-specific desensitization was also observed following much lower concentrations of acetylcholine and carbachol if the test drug was added without washing out the desensitizing concentration. This was normally done 4 min after the first addition, at which time with acetylcholine or carbachol there was a second contraction. No increase in tension could be obtained with acetylcholine or carbachol, and potassium either had no effect or caused a relaxation. These experiments reveal a non-specific desensitization having properties similar to the desensitization observed 1 min after washout of very high concentrations.

In the soleus muscle of the rat, the greater acetylcholine sensitivity occurs near the endplate and at the muscle-tendon junction; the remainder of the fibre has a detectable, though very low responsiveness (Miledi & Zelená, 1966; Albuquerque & Thesleff, 1968). After denervation, the entire fibre exhibits uniformly high sensitivity, comparable with that found in denervated fast muscles (Albuquerque & Thesleff, 1968). Assuming that the mouse is similar to the rat, the supersensitivity of the denervated soleus is due to an increase in receptor density in non-endplate regions and it can safely be assumed that contractions obtained in denervated muscles are initiated by interaction with these new receptors. Contractions obtained with high concentrations of acetylcholine in innervated soleus muscles might be caused by an action at receptors of the myoneural or muscle-tendon junctions or at the low density receptors covering the remainder of the muscle fibre. It is likely that it is the endplate receptors that are predominantly activated in these experiments, since similar small responses can be obtained in normal mouse diaphragms (M.A. Maleque, unpublished experiments) which are only sensitive to acetylcholine at the endplate (Miledi, 1960b; Hartzell & Fambrough, 1972).

Although desensitization was observed in chronically denervated and in innervated muscles, only in the latter was it found to be receptor specific. We have considered three explanations for this: (1) the properties of individual receptors in the two preparations may be fundamentally different, (2) specific desensitization may require intimate association of receptors with acetylcholinesterase or (3) the concentration of agonist may determine the type of desensitization produced. The first explanation seems to be ruled out since most comparative studies have revealed a striking similarity between receptors in the vicinity of the innervated endplate and in non-endplate regions of chronically denervated muscle (Axelsson & Thesleff, 1959; Freeman & Turner, 1969). Only quantitative differences have been demonstrated in sensitivity to antagonists (Jenkinson, 1960; Beránek & Vyskočil, 1967; Vyskočil & Beránek, 1968; Miledi & Potter, 1971; Lapa, Albuquerque & Daly, 1974) and in the acetylcholine reversal potential (Feltz & Mallart, 1971). Since receptor specific desensitization has been observed in cultured skeletal muscle (Harvey & Dryden, 1974a) which has no functional cholinesterase (Kano & Shimada, 1971; Harris, Marshall & Wilson, 1973; Harvey & Dryden, 1974b) the deficiency of cholinesterase in denervated muscle (Axelsson & Thesleff, 1959; Miledi, 1962; for review see Koelle, 1963) cannot be directly linked to the type of desensitization observed. We favour the third explanation: specific desensitization occurs only where the concentration of agonist is high, that is in those preparations that are insensitive. If a response is obtained with low doses, then it is impossible to achieve sufficient concentration to produce specific desensitization without causing such massive activation that non-specific changes occur, resulting in loss of sensitivity to a variety of agonists. This hypothesis is consistent with the Katz & Thesleff (1957) model for specific desensitization:

$$\begin{array}{ccc} A + R & \rightleftharpoons & AR \\ \uparrow \text{slow} & & \downarrow \text{slow} \\ A + R' & \rightleftharpoons & AR' \end{array}$$

A high concentration of agonist activates a greater fraction of receptors and thus drives a greater proportion of them into the desensitized configuration. Experimental evidence for greater desensitization at higher agonist concentrations has been obtained by Katz & Thesleff (1957), Miledi (1960a), Katz & Miledi (1964), Nastuk *et al.* (1966) and Harvey & Dryden (1974a).

M.A.M. was supported by an academic staff scholarship from the Association of Commonwealth Universities.

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(Received May 9, 1975. Revised June 9, 1975.)

Br. J. Pharmac. (1975), 55, 133-139

A COMPARISON OF THE EFFECT OF MESCALINE ON ACTIVITY AND EMOTIONAL DEFAECATION IN SEVEN STRAINS OF MICE

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1 Mescaline hemi-sulphate (35 mg/kg body weight) was injected intraperitoneally into male mice (*Mus musculus*) from seven genetically diverse laboratory strains.

2 The effect of mescaline was found by comparison of the emotional defaecation and open field activity of mice after mescaline injection with the performance of the same mice after a subsequent saline (0.9% w/v NaCl solution) control injection.

3 In strains A2G, C3H/He, C57BR/cd, CBA/Cam and F/St, mescaline inhibited emotional defaecation and stimulated open field activity. These effects did not occur in strains ICFW and Schneider.

4 A positive relationship was found between the degree of emotional defaecation characteristic of each strain in the saline control experiment and the inhibitory effect of mescaline on emotional defaecation.

5 Pre-treatment of mice with tranylcypromine (20 mg/kg body weight, i.p.) had no effect on emotional defaecation or on its inhibition by mescaline.

Introduction

The genetic resources of the wide variety of strains of the laboratory mouse (*Mus musculus*) have not been much exploited by pharmacologists. One might expect to gain valuable information on the relationship between the different effects of a single drug, or the similar effects of different drugs, by the use of genetic strains which differ in their pharmacological reactions. In spite of the good example set by pharmacogeneticists (Meier, 1963), in much pharmacological work on mice there seems to be an assumption that it is of no great consequence which strain of mice is used, provided it is albino. Indeed, the fascination of the albino gene is so great that some authors regard a phrase such as 'adult male albino mice' as a sufficiently scientific description of the strain they used and give no further details. In the belief that different strains of mice may differ profoundly in their reactions to drugs, I have measured some effects of mescaline on mice from seven different strains. Mescaline has been reported to have a variety of different effects on mice. These include twitching of the hindquarters (Cooper & Walters, 1972) or of the head (Corne & Pickering, 1967), inhibition of aggressiveness (Rewerski, Kostowski, Piechocki & Rylski, 1971), increased motor activity (Shah & Himwich, 1971), decreased motor activity (Cooper & Walters, 1972) and a modification of nest-building behaviour (Schneider & Cheroweth, 1970). I chose to study a version of open field activity, and 'emotional' defaecation. Male mice only were used in order to reduce the biological variables. The scheme was to inject each mouse with mescaline in one experiment, and with saline (0.9% w/v NaCl solution) in a subsequent control experiment, and see if the effect of mescaline showed any interesting differences in different strains.

Methods

The mice and their preparation

Mice from the following seven strains were used: A2G, C57BR/cd, C3H/He, F/St, ICFW (all from the Laboratory Animals Centre, Carshalton), CBA/Cam (from the Department of Genetics, University of Cambridge), and Schneider (from the Imperial Cancer Research Fund Laboratories, London). Strains A2G, ICFW and Schneider are albino. All the strains except Schneider are inbred. Adult (from 12 to 32 weeks old) males were routinely kept in cages (11 cm x 29 cm) with two females which were usually of the same strain as the male. Young litters were sometimes present. All males were kept in their respective cages for at least two weeks before being tested. The cage floors were covered with beechwood sawdust and the bedding was paper. Water and food (Christopher Hill No. 41B pencils) were supplied *ad libitum.* The ambient temperature of the animal house and the experimental laboratory varied between 19 and 23° C. Mice were left undisturbed during the day before a test and their cages were then brought to the laboratory and left undisturbed for at least 30 min before the test began.

The experimental schedule

The open field consisted of an 87.5 x 87.5 cm square of formica ruled into 49 squares. It was surrounded by a 35 cm high wall of brown cardboard and in the middle was placed an open-topped white cardboard starting enclosure 17 cm high and exactly enclosing the central square. The main illumination apart from diffuse daylight came from an Atlas 80 W 'Daylight' fluorescent tube 1.5 m above the open field. The noise level varied between 50 to 55 dB and came mainly from a window fan. Before each mouse was introduced, the open field and the starting enclosure were cleaned with dilute vinegar and wiped dry in order to remove (for the human nose) any smell left by the previous occupant. A mouse to be tested was gently transferred from its cage to the starting enclosure and allowed to walk off the hand on to the formica surface. After 3 min the enclosure was removed and the mouse left to explore the open field for 2 minutes. The number and the total weight of the faecal boluses produced during the initial 3 min and the number of squares in the open field subsequently entered were recorded.

The complete experimental schedule for each mouse began with the procedure described above. The mouse was then removed from the open field, weighed and injected intraperitoneally with mescaline hemi-sulphate (Sigma Ltd.) 35 mg/kg in saline. The volume injected was 0.1 ml per 10 g body weight. The mouse was then returned to its cage and the test repeated (without any injection) 1 h later, and 1 h later again. As a control the schedule was then repeated with the same mice between 7 and 10 days later, injecting normal saline in place of mescaline. Two batches of six males (each male kept with two females) were tested from each strain. Three males of each batch were tested in the forenoon and three in the afternoon. Data from all seven strains were collected in the course of one year and the testing of the two batches from each strain was separated by at least three months. The Walsh two-tailed test for paired non-parametric data (Siegel, 1956) was used to calculate the significance of the effect of mescaline on defaecation and open field activity. To find the effect of a monoamine oxidase inhibitor some mice were injected intraperitoneally with tranylcypromine (Smith, Kline & French) 20 mg/kg in saline 16 to 24 h before the mice were tested as described above.

Results

The total number of boluses produced by each batch of six mice in the mescaline experiment and in the subsequent saline control experiment are shown in Table 1. In the first five strains mescaline inhibited defaecation, moreover the degree of inhibition of the two batches within each strain was in no case significantly different. (The second batch of A2G and C3H mice were pre-treated with tranylcypromine but since this had no significant effect on their defaecation or on its inhibition by mescaline they have been included in the general comparison. Tranylcypromine had been expected to modify the effect of mescaline for reasons which are explained later.) With ICFW and Schneider mice the number of boluses produced after mescaline was not significantly different from the number produced after saline, and there was therefore no evidence of any effect of mescaline in these two strains. However, it is clear that ICFW and Schneider mice defaecate much less than the other five strains, and it is arguable that they defaecate so little that their occasional defaecation is not an emotional reaction but a physiological necessity which is not amenable to inhibition by mescaline. This would mean that some other measure of the effect of mescaline is needed for these two strains, and such a measure is fortunately provided by open field activity.

Table 2 shows the mean activity of each strain before injection and 1 h after injection with mescaline or saline. Two hours after injection the effect of mescaline on activity was much reduced, and more variable. The data from both batches have been pooled in all strains except A2G and C3H. In most strains there was an increase in activity after mescaline and a decrease in activity after saline, the effect of mescaline is therefore calculated as shown in Table 2. With ICFW and Schneider mice mescaline was followed by reduced activity, indeed in both these strains the reduction in activity after mescaline was not significantly different from the reduction in activity after saline. Therefore with both defaecation and activity measurements the conclusion is that mescaline had no measurable effect on ICFW or Schneider mice. In C57BR, CBA and F/St mice the effect of mescaline was a significant increase in activity. In batch 1 of both A2G and C3H

Table 1 Numbers of faecal boluses produced by each batch of six mice in each test

Strain:	*A2G*		*C57BR/cd*		*C3H/He*		*CBA*		*F/St*		*ICFW*		*Schneider*		*Totals*
Batch:	*1*	*2**	*1*	*2*	*1*	*2**	*1*	*2*	*1*	*2*	*1*	*2*	*1*	*2*	
Mescaline experiment															
Before injection	20	25	11	18	17	17	23	20	21	22	0	0	3	0	197
1 h after injection	0	3	6	0	4	7	4	0	4	8	0	0	1	1	38
2 h after injection	5	4	3	9	3	4	11	10	10	6	2	3	4	1	75
Total after mescaline	5	7	9	9	7	11	15	10	14	14	2	3	5	2	113
Saline experiment															
Before injection	22	29	14	27	19	13	19	22	18	20	0	0	3	0	206
1 h after injection	24	25	15	18	16	17	9	10	16	13	2	1	3	0	169
2 h after injection	26	20	22	23	25	18	17	16	11	15	4	4	4	0	205
Total after saline	50	45	37	41	41	35	26	26	27	28	6	5	7	0	374
†Effect of mescaline	45	38	28	32	34	24	11	16	13	14	4	2	2	−2	
Probability less than	0.011		0.011		0.011		0.011		0.048		NS		NS		
Mean age at mescaline expt. (wks)	24	26	21	19	27	30	20	25	25	24	30	18	28	21	
Mean body weight (g)	24	24	21	26	27	27	21	25	16	20	29	24	29	33	

* Pre-treated with tranylcypromine. † The total number of boluses after saline minus the total after mescaline. NS = not significant.

Table 2 Mean numbers of open field squares entered

Strain:	*A2G*		*C57BR/cd*	*C3H/He*		*CBA*	*F/St*	*ICFW*	*Schneider*	*Totals*
Batch:	*1*	*2**	*1 & 2*	*1*	*2**	*1 & 2*	*1 & 2*	*1 & 2*	*1 & 2*	
						Mescaline experiment				
Before injection (A)	22	6	78	50	28	26	52	41	88	391
1 h after injection (B)	40	20	104	62	42	51	49	32	61	
						Saline experiment				
Before injection (C)	25	14	90	52	40	41	48	68	69	447
1 h after injection (D)	5	13	59	40	41	27	22	48	49	
Effect of mescaline (B–A) + (C–D)	38	15	57	24	13	39	23	11	–7	
s.e. mean	10.9	6.9	9.6	7.3	13.3	10.5	6.1	7.5	8.9	
Probability less than	0.094	0.094	0.011	0.031	NS	0.011	0.011	NS	NS	
	0.011			0.048						

* Pre-treated with tranylcypromine. NS = not significant. s.e. mean = standard error of the mean of the effect of mescaline

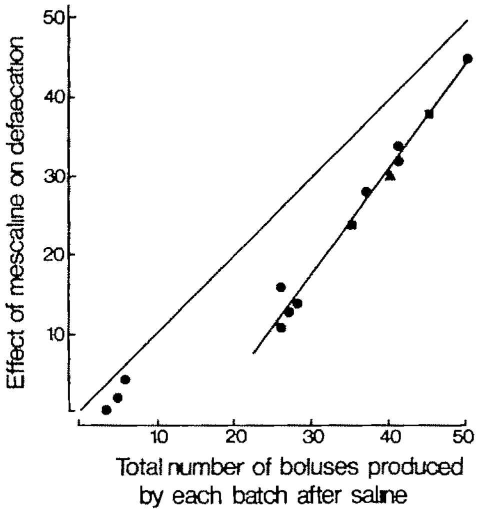

Figure 1 Display of data in Table 1 to show the relationship between defaecation after saline and the effect of mescaline on defaecation. Each symbol represents one batch of six mice, except in the Schneider strain in which the two batches have been pooled and then halved to give an average value for a batch of six Schneider mice. (▲) CBA batch 3; (■) tranylcypromine-treated batch 2 of A2G and C3H/He.

mescaline also increased activity significantly. In both the tranylcypromine-treated second batches of A2G and C3H the effect of mescaline was less marked although not significantly different from the untreated batches. The effect of tranylcypromine was not investigated further. Among the five strains in which mescaline had an effect on both activity and defaecation there was no correlation in the size of the effect on the two measurements. This is not surprising when one reflects that open field activity is a more complex activity than defaecation, and the effect of mescaline on activity is therefore subject to a wider range of influences, both genetic and environmental, than is its effect on defaecation. Nevertheless it seems that the ICFW and Schneider mice are so much less responsive to mescaline than are the other five strains that the difference can be seen in both the defaecation and the activity measurements. The totals on the right of Table 2 show that the mice were on average less active before the mescaline injection than they were before the subsequent saline injection, however the tendency was not statistically significant (Walsh test, $P > 0.1$).

If the effect of mescaline on defaecation is examined in greater detail (Table 1) among the five high-defaecating strains an inverse relationship between the number of boluses produced after mescaline and after saline can be discerned. This relationship means that the inhibitory effect of mescaline is greatest in those strains which defaecate most after saline. This is shown in Figure 1, in which a batch which was completely inhibited by mescaline would be plotted on the diagonal line drawn from the origin. The greater the number of boluses produced by a batch after mescaline the greater the vertical distance it falls below the diagonal. The two batches in each strain fall fairly near to each other along the regression line ($b = 1.35$, $P < 0.001$) which is to be expected if genetical factors are important in determining their position. The ICFW and Schneider data clearly do not fall on the regression line, but as we have already seen they can be regarded as extreme examples of the same tendency in that they defaecated very little after saline and were not significantly inhibited by mescaline. These two strains each defaecated significantly less after mescaline than any of the other five strains except A2G. The regression line in Figure 1 cuts the abscissa scale at 16.5 boluses. One would therefore expect mescaline not to inhibit strains which produced less than about 16 boluses after saline under the conditions used in these experiments.

Effect of dosage Perhaps the few boluses produced by ICFW and Schneider mice were not amenable to inhibition by mescaline, even at a higher dosage. This possibility was not tested, but a dose-response experiment was carried out with batch 2 from each of strains C57BR and CBA. These were the two available batches which had previously given the highest post-mescaline defaecation scores and

Table 3 The effect of dosage on inhibition of defaecation by mescaline

Dosage of mescaline (mg/kg)	*17.5*	*35.0*	*70.0*
		C57BR/cd (Batch 2)	
Before injection	18	18	20
1 h after injection	1	0	0
2 h after injection	9	9	2
Total after mescaline	10	9	2
		CBA (Batch 2)	
Before injection	18	20	21
1 h after injection	7	0	3
2 h after injection	9	10	4
Total after mescaline	16	10	7
Pooled totals	26	19	9

(The data for the standard dose of 35 mg/kg are taken from Table 1) which were therefore expected to respond most readily to an increase and a decrease of mescaline dosage. C57BR batch 2 was tested with mescaline at half the normal dosage and subsequently at twice the normal dosage. CBA batch 2 was tested with the same dosages but in the reverse order. The results are shown in Table 3 where it can be seen that each batch responded to the changes in dosage. The effect is statistically significant if the data from both batches are pooled ($\chi^2 = 8.10$, $P < 0.025$).

Validity of saline control experiment

Another possibility to be considered was that mescaline affected defaecation in the saline experiment several days later. In order to test this possibility a third batch of each of the A2G, CBA and F/St strains was set up and taken through the standard experiment with saline. The results are shown in Table 4. The A2G and F/St data agree well with those in Table 1 and support the assumption that the mescaline experiment had no effect on the result of the subsequent saline experiment. The CBA batch 3 gave a total which was much higher than expected. However, when this CBA batch was then tested with mescaline it gave a total of only 10 boluses after injection, which brings it close to the regression line (see Figure 1). The open field data from these three controls were not significantly different from the data of mice previously used in a mescaline experiment.

Bolus weight

In nearly all batches throughout the whole series of experiments described above, the mean bolus weight increased slightly after mescaline injection and decreased slightly after saline injection.

Discussion

The data obtained in these experiments describe a relationship between a normal behaviour in seven strains of mice and the effect of mescaline on that behaviour. Let us suppose that when a mouse is put into the enclosure the immediate result is a release in the body of a chemical substance, perhaps an amine, which predisposes the mouse to defaecate if it rises to a sufficient concentration in some part of the brain. Let us also suppose that the hypothetical amine is released in approximately the same amount in mice of different strains. Now suppose that there is an enzyme which inactivates the amine, and that the enzyme is present in greater activity in some strains than in others. In the strains with high enzyme activity the amine will be rapidly inactivated and therefore prevented from reaching an effective level. In the strains with lower enzyme activity the amine will not be inactivated fast enough to prevent it from reaching an effective level and defaecation will take place. The lower the enzyme activity the more amine will accumulate and the more defaecation will take place. On this hypothesis A2G would have the lowest enzyme activity and Schneider the highest activity. Mescaline is metabolized in the mouse and excreted as a mixture of various metabolites and unchanged drug (Shah & Himwich, 1971). If the same enzyme that inactivates the hypothetical defaecation amine also plays an important part in the inactivation of mescaline, this would explain why the effect of mescaline was greater in the higher-defaecating strains and least in the two lowest-defaecating strains.

Table 4 Numbers of boluses produced in saline experiment by mice which had not previously been injected with mescaline

Strain: *Batch*	*A2G* *3*	*CBA* *3*	*F/St* *3*
Before injection	23	19	13
1 h after injection	27	21	15
2 h after injection	26	19	9
Total after saline	53	40	24

Which enzyme might be involved? There is some evidence that monoamine oxidase (MAO) can deaminate mescaline in the mouse (Zeller, Barsky, Berman, Cherkas & Fouts, 1958; Shah & Himwich, 1971). It was for this reason that the second batches of A2G and C3H mice were pre-treated with tranylcypromine before being tested with mescaline or saline. As it turned out these two strains were ill-chosen because the hypothesis predicts that they are relatively deficient in MAO (or some other enzyme) and one would therefore not expect them to be much affected if their MAO were inhibited. Unfortunately one would expect the same result if MAO had nothing to do with defaecation. ICFW or Schneider mice would have been a better choice, however, a smaller trial of tranylcypromine on Schneider mice also had no effect on defaecation either before or after mescaline injection, and one must conclude that MAO is probably not involved. There is good evidence (Zeller *et al.*, 1958) that diamine oxidase can deaminate mescaline in several species, and it would be interesting to see if a diamine oxidase inhibitor such as aminoguanidine (Kapeller-Adler, 1970) has any effect on emotional defaecation in ICFW or Schneider mice.

As an alternative hypothesis one could suppose that the genetical variation between strains is not due to variation in the activity of an enzyme but to variation in the number of available receptor sites. If the hypothetical defaecation amine and mescaline share a common receptor site in the brain then genetical variation in the number of such sites available would be expected to alter the response to both amines in the same direction.

It is clearly imperative that pharmacological work on mice should be done with genetically defined strains. No statement about the species as a whole can be reasonably made until several different strains have been investigated. There is no reason yet to suppose that other experimental animals are less genetically variable than the mouse, with the possible exception of the laboratory populations of the Syrian Hamster (*Mesocricetus auratus*) which are apparently all descended from one breeding pair (Searle, 1968).

Mescaline is not an addictive drug. However, where addiction to a certain drug is thought to be heritable, the method of comparing several different inbred strains with respect to both addiction and also possible biochemical causes of addiction might well be informative.

I am grateful to Dr M.B.H. Youdim for the tranylcypromine, to Miss Jill Ford, A.I.A.T., for her care of the mice, and to the Medical Research Council for financial support.

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(Received April 15, 1974.
Revised April 25, 1975.)

Br. J. Pharmac. (1975), 55, 141-150

TIMID SINGLY-HOUSED MICE: THEIR VALUE IN PREDICTION OF PSYCHOTROPIC ACTIVITY OF DRUGS

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1 About 45% of singly-housed male mice showed timidity (alert postures, running away, defensive postures) instead of aggression on interactions in pairs with group-housed male mice, though their partners did not show any aggression. The isolation-induced timidity was stable in repeated interactions. Timid mice also showed locomotion (walking across cage and rearing) and a small amount of sociable activity (sniffing, following partners and climbing over them).

2 Diazepam (5 mg/kg), chlordiazepoxide (20 mg/kg), chlorpromazine (7.5 mg/kg) and barbitone (60 mg/kg) given orally inhibited the isolation-induced timidity without reducing other motor activities in the timid mice. Imipramine lessened timidity only in a dose (80 mg/kg) which also decreased other components of behaviour in the timid isolates. (+)-Amphetamine and lysergic acid diethylamide (LSD) increased the timid response.

3 Comparison of the inhibition of timid activities with changes in other behaviour occurring at the same time seems a better measure of selective timidity-reducing effects of drugs than the rota-rod test.

4 Diazepam (5 mg/kg) increased sociable and locomotor activities. Barbitone (20 and 60 mg/kg) increased sociable activities; however, the higher dose also evoked some aggression in timid mice.

5 Behaviour of timid singly-housed male mice seems to be a good measure for prediction of activity of drugs in relieving anxiety as well as for detection of aggression-evoking and sociability-increasing effects of drugs.

Introduction

It is well-known that individually-housed male mice can show defensive-escape activity as well as aggressive behaviour on interaction with strange males (Scott & Fredericson, 1951). The defensive-escape activity of singly-housed mice has been considered to be a mere passive submissive response coerced by the more aggressive partner. However, it has been found recently (Cairns & Nakelski, 1971; Kršiak & Borgesová, 1973) that some male mice housed singly for several weeks show alert and defensive postures, exaggerated escape responses and squeaking instead of aggression even on interaction with completely non-aggressive male mice. The first aim of the present paper was to discover whether isolation-induced timidity in mice can be used as a measure of a drug's effectiveness in relieving anxiety.

It has always been difficult to assess the relative specificity of behavioural actions of drugs in animal models. Usually some measure of 'neurotoxicity' such as decreased spontaneous motor activity, ataxia, loss of righting reflex, disruption of rotating rod performance are used for assessing the selectivity of the action of a drug on behaviour. However, there is still no established rationale for selection of different neurotoxicity measures as reference effects for judging this specificity (Cook & Kelleher, 1963). Furthermore, neurotoxicity is ascertained mostly in different animals with another history and in other experimental conditions which can greatly change their sensitivity to a drug. Therefore, an attempt was made in the present paper to assess the selectivity of the timidity-reducing effect of drugs by comparing this with effects on other components of behaviour occurring in the same animals and tests. The rota-rod test, which is a common measure of neurotoxicity in experiments on singly-housed mice (e.g. Janssen, Jageneau & Niemegeers, 1960; Sofia, 1969; Valzelli, 1973) has also been used.

Apart from the defensive-escape activities, timid isolates show locomotion (walking across cage and rearing), a small amount of sociable activity (sniffing and following partners and climbing over them), and after repeated interactions some aggressive behaviour. The third aim of the present paper was to ascertain the value of non-timid behavioural activities in assessing the psychotropic activity of drugs.

Methods

Subjects, housing and apparatus

Male albino random-bred Swiss mice weighing 18-20 g at the beginning of the experimental housing were used. They were housed singly in self-cleaning cages or in groups of 20. The cages used for the individual housing had solid metal walls 13 cm high with wire-mesh floors (8 x 16 cm) which were placed on trays with wood shavings. Except on experimental days, the isolates were not handled throughout the isolation period. The mice kept in groups were housed in standard plastic cages 25 cm high with solid bottoms (22 x 38 cm) covered with wood shavings. All mice were housed in a natural day-and-night cycle at temperatures ranging from 22 to 24°C. Food and water were available permanently *ad libitum*.

The mice were observed in transparent cages (20 x 30 x 20 cm) with wood shavings on the floor and open tops. The observations were performed in a quiet experimental room from 08 h 30 min to 16 h 00 min under moderate artificial dispersed lighting.

Procedure

Social interactions were started after 3-5 weeks of isolation, always involving one singly-housed and one group-housed mouse in the observational cages. The isolates were allowed 30 min adaptation in the observational cages before the group-housed partners were introduced; interactions ended after 4 minutes. The observational cages were cleaned and their floors were covered with new wood shavings after each interaction.

Altogether 3-5 interactions were repeated one week apart with 355 pairs of singly- versus group-housed mice. Each isolate was paired with the same group-housed partner throughout the whole experiment. The isolates were given drugs or water orally 30 min before each interaction in a randomized order according to the Latin square design (each mouse served as its own control). The Wilcoxon matched pairs signed-ranks test (Siegel, 1956) was used for statistical evaluation. The group-housed mice were given only water.

Measures

The incidence of the following behavioural acts and postures similar to those described by Grant & Mackintosh (1963) was recorded by a keyboard-counter system:

Sociable activities: Social sniff—sniffing the partner's head, body, genitals or tail. *Climb*—the mouse places its forepaws on the partner's back, mostly in the shoulder region, and usually sniffs this area at the same time (Grant & Mackintosh called this Attempted Mount). *Follow*—following the partner by a quiet walking.

Timid activities: Alert posture—a sudden interruption of all movements with eyes and ears being directed towards the other mouse (Attend). *Escape*—a rapid running or jumping away from the opponent (Retreat and Flee). *Defence*—the mouse responds to the partner's social behaviour by raising the forepaws, hunching the back or by rearing up on the hind legs with the head up and forelegs extended (Defensive or Submissive upright posture).

Aggressive activities: Attacks—a fierce lunging at the partner from various sides often associated with biting. *Aggressive unrest*—walking around the partner (Walk round, mince) or on its own axis (Circle) walking to and from the partner (To-fro) and chasing the partner. *Tail rattle*—rapid vibrations of the tail were classified as an ambivalent activity reflecting both aggressive and flight tendency.

Locomotion (non-social activities): *Walk across cage*—any walking which is apparently not related to the partner. *Rear*—the mouse stands only on his hind legs and usually sniffs air or walls at the same time.

The interobserver reliability of the recorded items was satisfactory, as determined by two observers recording independently behaviour of 18 mice in interactions lasting 200 seconds. The r_S values ranged from 0.7 to 0.8. Observers did not know which kind of treatment was given to the tested animals.

Apart from the listed items, five other activities were also observed in the tested animals: approach (walking to the partner), leave (walking away from the partner), crouch (all the ventral surface from the chin to the tail is pressed to the floor, with the back moderately hunched and eyes semiclosed or closed while the other mouse usually climbs at the same time), self-groom (the mouse licks or scratches its body or wipes its face with forepaws) and resting (the mouse sits quietly on all fours, moves occasionally with the head or forepaws without orientation to the partner). However, these activities were not included in the present results for the following reasons: approaching

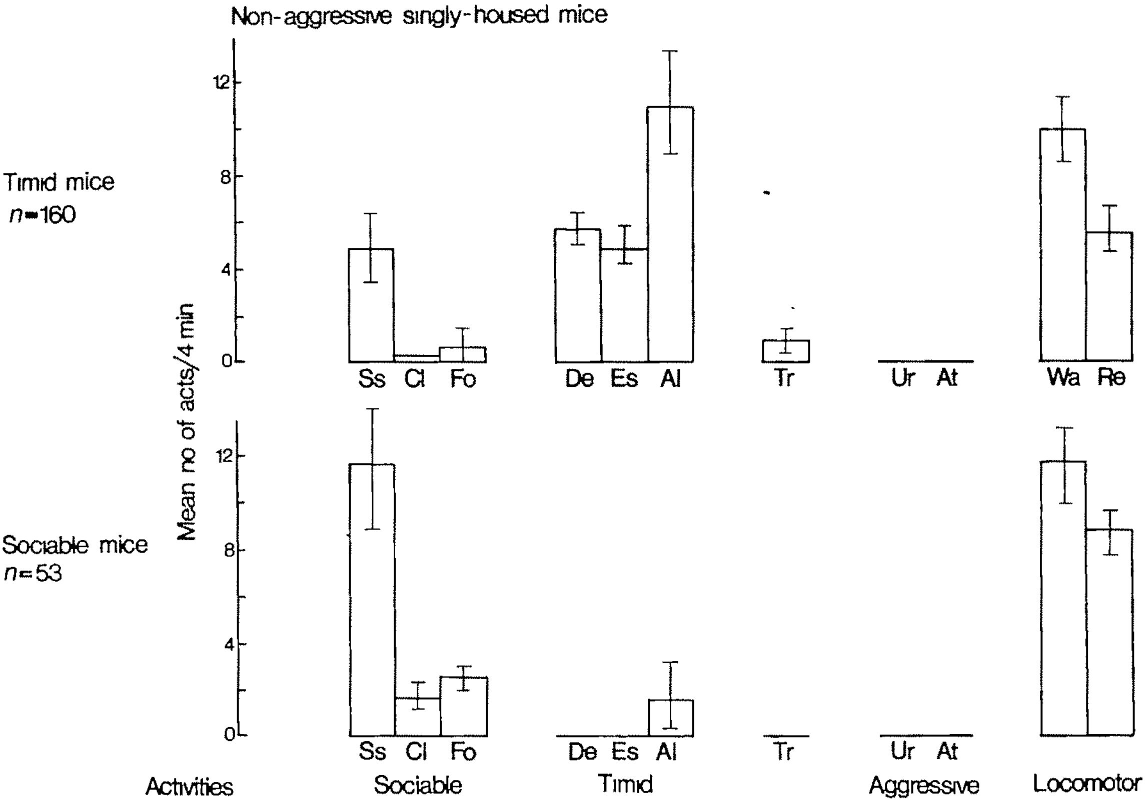

Figure 1 Behaviour of non-aggressive singly-housed male mice in the control paired interaction with non-aggressive group-housed male mice. Code for abbreviations: Ss = social sniffing, Cl = climbing over partner, Fo = following partner, De = defensive posture, Es = escape, Al = alert posture, Tr = tail rattling, Ur = aggressive unrest, At = attack, Wa = walking across cage, Re = rearing.
Limits of confidence of means for $P = 0.05$ are given.

partner or leaving him were difficult to classify (they occurred in a context of sociable as well as of aggressive activities while after higher doses of amphetamine they represented a mere stereotype walking), length of a bout of resting varied greatly after drugs so that the incidence of rests was not a reliable indicator of the actual amount of resting, and finally, crouching and self-grooming occurred very infrequently and their incidence was not increased by any drug tested.

Rota-rod test

Mice were trained to maintain themselves on a slowly revolving rod (4 rev/minute). The surface of the rod (diameter 2 cm) was rough. Mice were tested 30 min after oral administration of drugs. The drug was considered effective when the animal fell from the rod more than once during a 1 min testing period. Mean effective doses and relative potencies of drugs were estimated according to the graphic probit method (Litchfield & Wilcoxon, 1949).

Results

Behaviour of non-aggressive isolates in control interactions

About 60% of isolates did not attack their partners in the control interaction. The non-aggressive isolates were classified in two groups (Figure 1): a large group of males exhibiting defence postures or escapes ('timid' mice representing 45% of all isolates, $n = 160$) and a small group showing a higher number of sociable activities ('sociable' mice, 15%, $n = 53$). The defensive-escape activity of the isolates was not a passive response to aggressive behaviour of their partners. Group-housed males interacting with timid isolates did not attack them nor did they show any apparent

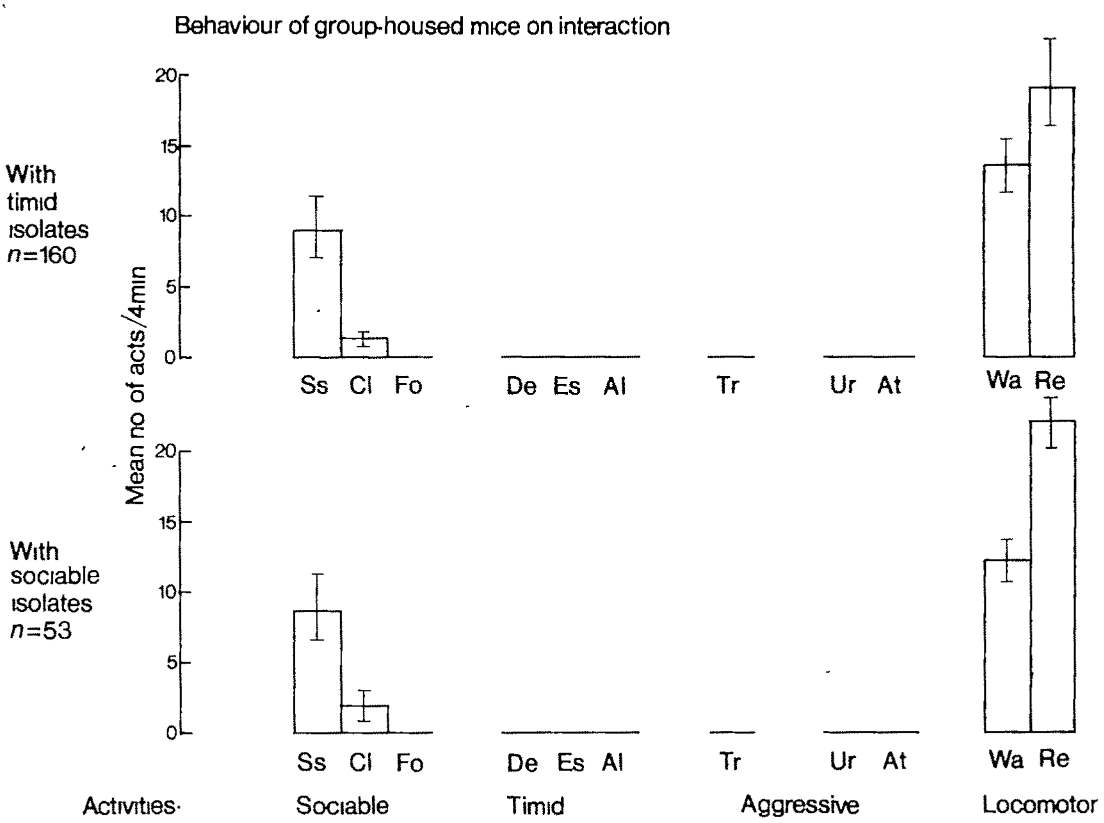

Figure 2 Behaviour of group-housed mice in the control paired interaction with timid or sociable singly-housed mice. Code for abbreviations is the same as in Figure 1. Limits of confidence of means for $P = 0.05$ are given.

aggressive activity (Figure 2). In fact, the behaviour of group-housed mice interacting with timid males did not differ from that of the group-housed mice interacting with sociable isolates. This suggests that the defensive-escape activity is provoked in isolates by the very presence of the strange partner and is therefore termed isolation-induced timidity.

The isolation-induced timidity was stable in repeated interactions: the correlation between the number of timid activities in the even and odd interactions in 47 isolates was highly significant (Figure 3).

The incidence of timid activities gradually decreased upon repeating interactions, particularly when the inter-trial intervals were as short as 2-4 days (Kršiak & Borgesová, unpublished results).

The rest of the isolates (about 40%, $n = 142$) which attacked their partners in the control interaction are not included in the present study.

Effect of drugs on behaviour of timid isolates

Diazepam (5 mg/kg, orally) and chlordiazepoxide (20 and 50 mg/kg) significantly lowered timid activities without reducing other components of behaviour (Figure 4). Diazepam seemed to stimulate rather than to inhibit the timid isolates: their sociability, walking across cage and total activity were significantly increased. Similarly, the timidity-reducing dose of barbitone (60 mg/kg) also increased sociability and total activity in the timid isolates (Figure 5). However, in contrast to diazepam, barbitone (60 mg/kg) stimulated aggressive activities. A lower dose of barbitone (20 mg/kg) significantly increased only sniffing and following partners.

Though the dose of chlorpromazine (7.5 mg/kg) inhibiting timidity did not significantly reduce another activity separately, a decrease was found when all activities were

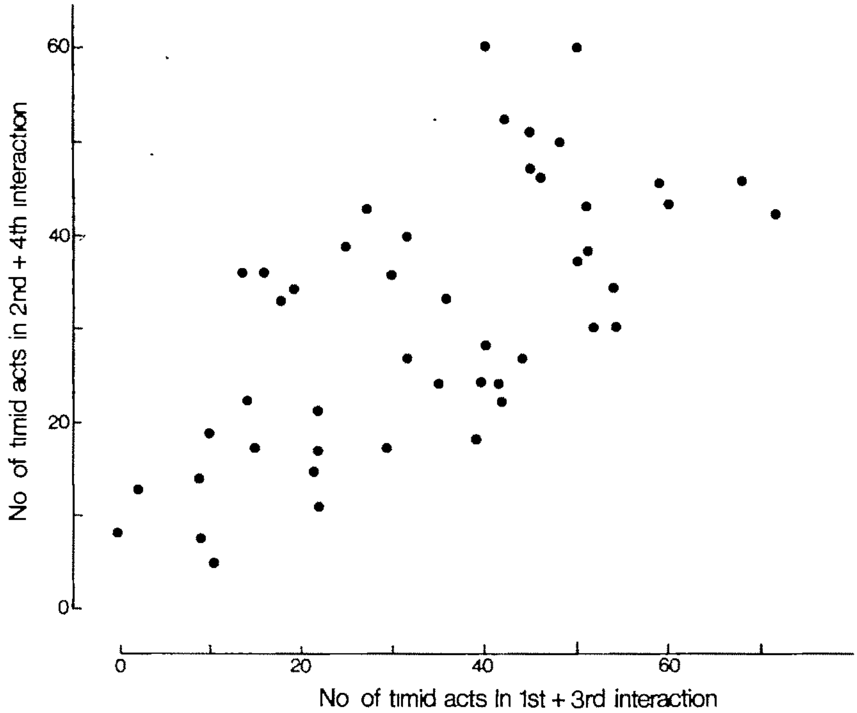

Figure 3 Correlation of occurrence of timid activities in the odd and even interactions in 47 timid singly-housed mice paired with non-aggressive group-housed mice. The interactions lasted 4 min and were repeated at weekly intervals. $r_S = 0.66, P < 0.001$.

combined, which suggests a general depressant effect of this dose of chlorpromazine (Figure 5). A lower dose of chlorpromazine (2.5 mg/kg) was ineffective.

Imipramine significantly reduced timid activities only in a high dose (80 mg/kg) which decreased walking across cage and rearing as well as all activities combined.

(+)-Amphetamine and LSD (Figure 6) did not reduce timid activities to any extent in any dose tested (0.25-4 mg/kg or 0.01 and 1 mg/kg respectively). On the contrary, 0.25 mg/kg of (+)-amphetamine significantly increased the number of alert postures and LSD (0.01 and 1 mg/kg) significantly stimulated tail-rattling. (+)-Amphetamine reduced sociable activities while LSD produced rather a general increase of all activities in the timid mice.

Inhibition of rota-rod activity

Mean effective doses of diazepam, chlordiazepoxide, barbitone, chlorpromazine and imipramine that induced falling off the rota-rod were lower in singly-housed mice than those in group-housed mice (Table 1). However, the ability to hold the rota-rod was smaller in isolates than in group-housed mice even when they had received no drugs: only 9 of 25 isolates remained on the rota-rod after 3 min in contrast to 19 of 24 group-housed males ($X^2 = 9.32$, $P < 0.005$). The untreated isolates seemed to be restless and frequently jumped from the rota-rod soon after being put on it.

Discussion

Isolation-induced timidity as the spontaneous tendency to withdraw from the non-aggressive partner does not seem to be a frequently described phenomenon in mice for several reasons. Another type of interaction (isolated versus isolated mice) seems to have been more commonly used, where defence and escape mostly represent passive responses to attacks of more aggressive partners

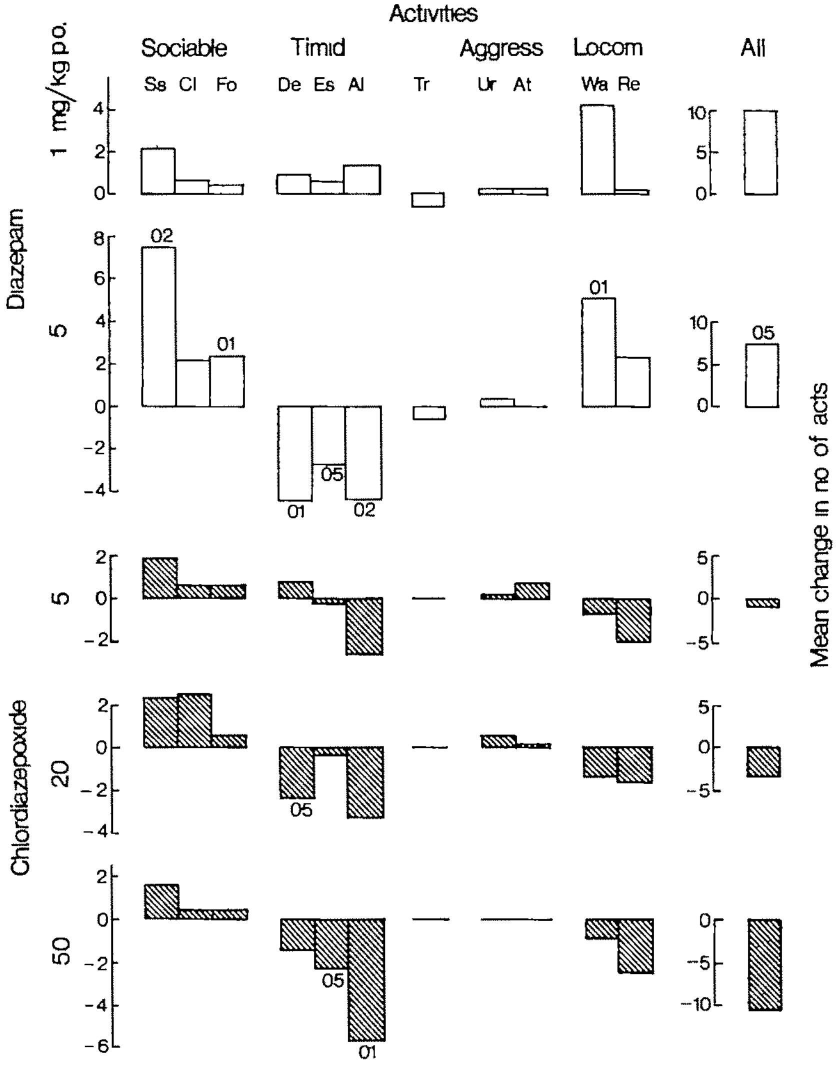

Figure 4 Behaviour of singly-housed timid mice given diazepam or chlordiazepoxide in paired interactions with non-aggressive group-housed mice. The ordinate scale shows the number of acts during 4 min expressed as the mean difference from activity in the control interaction (which did not differ significantly from that depicted in Figure 1). Effects of each dose represent mean results from 8 to 15 timid mice.

Table 1 Doses (mg/kg, orally) producing falling off the rota-rod

Drug	*Singly-housed mice ED_{50} 95% confidence limits*	*Group-housed mice ED_{50} 95% confidence limits*	P⁺
Diazepam	3.1 (2.0-4.8)	4.1 (2.7-6.4)	NS
Chlordiazepoxide	9.5 (6.6-13.8)	47.0 (33.1-66.7)	0.05
Barbitone	72.0 (48.0-108.0)	180.0 (86.7-195.0)	0.05
Chlorpromazine	5.0 (3.0-8.3)	8.8 (5.5-14.1)	NS
Imipramine	31.0 (23.1-41.5)	140.0 (101.5-193.2)	0.05

⁺ Level of significance of the difference between the two ED_{50} values. NS = not significant.

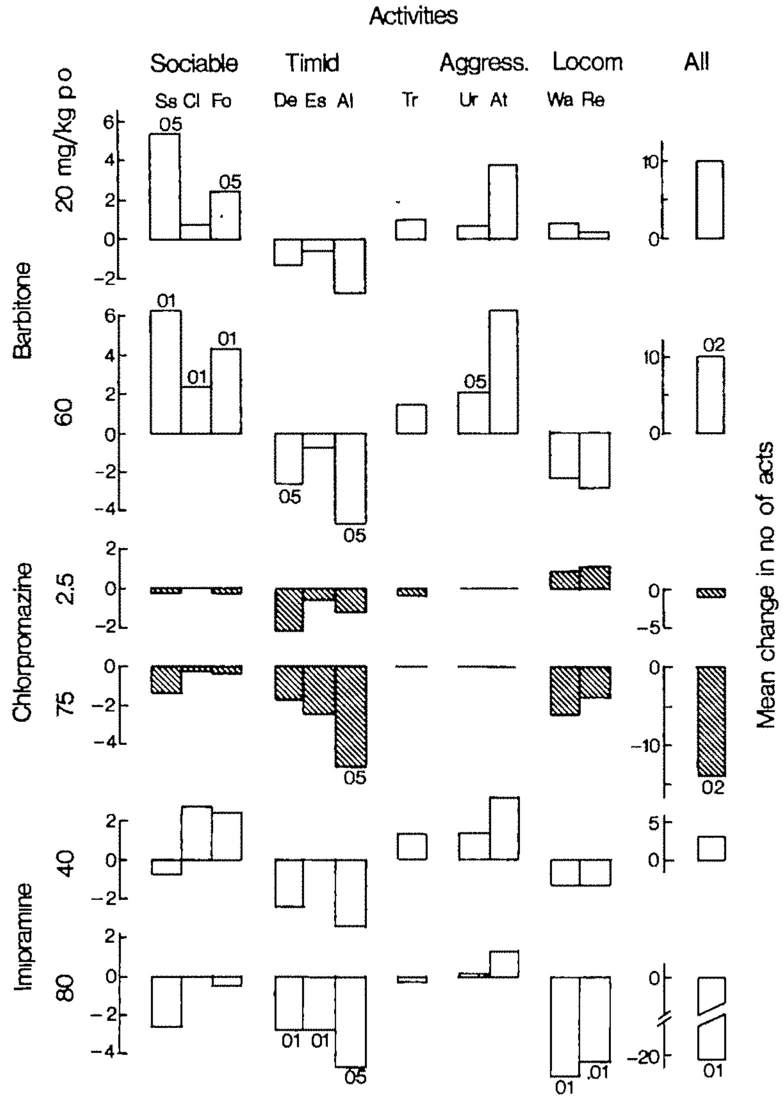

Figure 5 Behaviour of singly-housed timid mice given barbitone, chlorpromazine or imipramine in paired interactions with non-aggressive group-housed mice. Scale as for Figure 4. Effects of each dose represent mean results from 8 to 23 timid mice.

(e.g. Ginsburg & Allee, 1942: Valzelli, Giacalone & Garattini, 1967; Sofia, 1969). When the interaction of isolated versus group-housed mice was used, then in most cases only aggressive mice were selected (e.g. Yen, Stanger & Millman, 1959; Janssen *et al.*, 1960, DaVanzo, Daugherty & Kang, 1966). Nevertheless, Cairns & Nakelski (1971) observed that most isolated mice initially reacted by withdrawal, startle response or freezing to a mere approach or sniff by non-aggressive group-housed partners. These authors used C57 BL/10 mice, which suggests that the timid reaction is not limited to the strain used in the present study.

It is advisable to keep the aggressiveness and social activity of partners as low as possible, if the defensive-escape activity of singly-housed mice is to represent timidity rather than a passive submissive response. Since aggressiveness of male mice increases sharply as the number of males housed together decreases below ten (Welch & Welch, 1966), mice housed in groups of 20 were

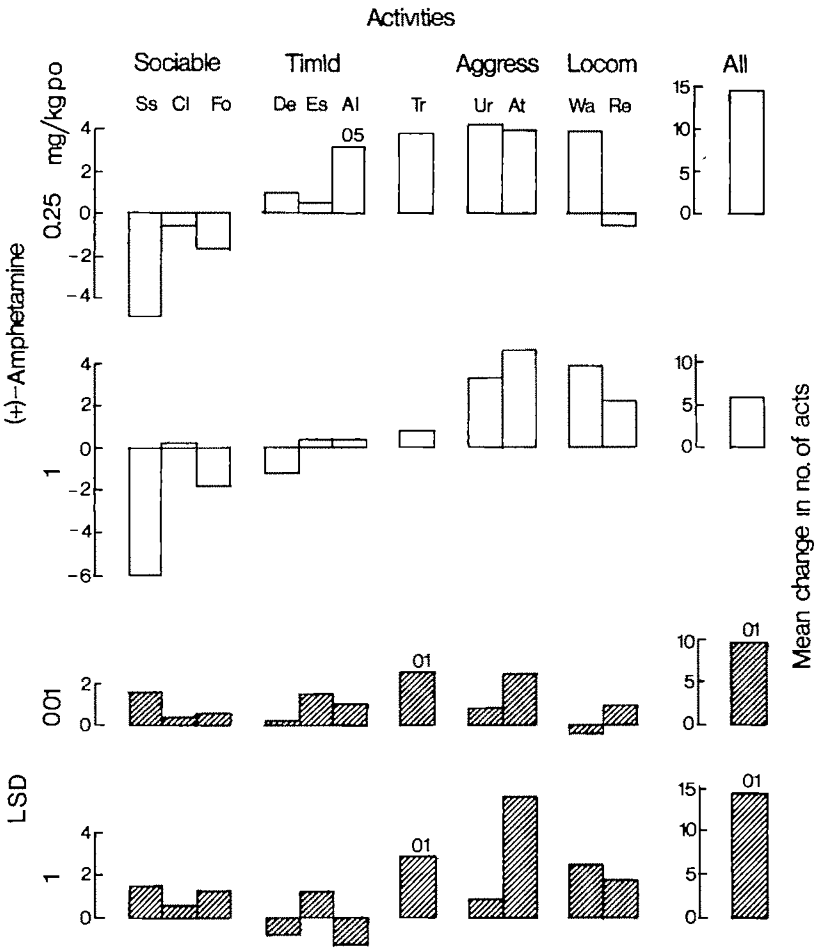

Figure 6 Behaviour of singly-housed timid mice given (+)-amphetamine or LSD in paired interactions with non-aggressive group-housed mice. Scale as for Figure 4. Effects of each dose represent mean results from 10 to 19 timid mice.

Table 2 Summary of experimental therapy of isolation-induced timidity in mice

Drug	*Lowest dose inhibiting timidity (LDIT) (mg/kg, p.o.)*	*'Adverse effects'*			*LD_{50} (mg/kg, p.o.)*	*LD_{50}/LDIT*
		'General sedation'	*'Muscular weakness'*	*Other*		
Diazepam	5.0	no	no	no	720^{+}	144
Chlordiazepoxide	20.0	no	no	no	620^{+}	31
Chlorpromazine	7.5	yes	no	no	319^{++}	43
Barbitone	60.0	no	no	incr. aggress.	600^{++}	10
Imipramine	80.0	yes	yes	no	400^{++}	5

(+)-Amphetamine increases timidity; LSD increases timidity.

'General sedation' = a significant decrease of all behavioural activities combined. 'Muscular weakness' = a systematic decrease of upright- or walk-type movements.

$^{+}$ Randall, Scheckel & Banziger, 1965; $^{++}$ Barnes & Eltherington, 1965.

chosen as partners of isolates. Furthermore, group-housed mice were not given opportunities to adapt themselves in the testing cage, so that their social activities appeared to be substituted by a strong tendency to explore the cage. As a result, group-housed mice never attacked timid isolates throughout the whole experiment.

Table 2 summarizes the evaluation of experimental therapy of isolation-induced timidity in mice. When the therapeutic efficacy is judged according to intensity of 'adverse' effects produced by the lowest 'therapeutic' dose in timid isolates, then diazepam and chlordiazepoxide appear to be 'drugs of choice' for the treatment of the mouse timidity (no adverse effects). Chlorpromazine and barbitone seem to be less suitable drugs because of sedative effects of chlorpromazine or increased aggressiveness after barbitone. Imipramine can be classified as quite unsuitable because of the marked toxicity of its effective dose, and amphetamine and LSD seem to be 'contraindicated' in the timid mice, as they tended to increase timidity. Accordingly, therapeutic efficacy of these drugs in the isolation-induced timidity in mice seems to be in good agreement with their effects in relieving anxiety in man.

When the therapeutic efficacy is judged by comparing lowest doses inhibiting timidity with some more commonly used measure of 'neurotoxicity', for instance falling off the rota-rod (e.g. Sofia, 1969; Valzelli, 1973), none of the tested drugs would reduce timidity at less than the 'neurotoxic' dose (Table 1). Mice rear on hind legs when displaying defensive postures and they run away when escaping. The reduced number of defensive postures and escapes after diazepam does not seem to be due to reduced ability to raise the front part of the body or to walk, as rearing and walking across cage were not reduced but increased in the diazepam-treated mice. Moreover, 5 mg/kg of diazepam stimulated sociability which represents a well-coordinated and directed activity. Therefore, the diazepam-reduced timidity does not seem to be due to a neuromuscular impairment as the low ED_{50} of diazepam for falling off the rota-rod might indicate.

Validity of the rota-rod test for assessing selectivity of behavioural effects of drugs seems to be questionable. If some animals spend less time on the rota-rod it does not invariably mean that these animals are less capable of a particular behavioural act (e.g. to escape, to attack, etc.). The untreated singly-housed mice were less able to maintain themselves on the rota-rod than the group-housed animals, yet, they exhibited complex motor activities on social interaction. Some benzodiazepines stimulate spontaneous locomotion in mice in doses that are many times higher than those producing falling off the rota-rod (Gluckman, 1971). The reduced ability to hold the rota-rod does not seem to reflect a unitary 'neurotoxic' change, since apart from ataxia or myorelaxation, it might be caused by an increased restlessness, e.g. by increased spontaneous jumping off the rota-rod. Furthermore, differences in housing conditions of animals used for the rota-rod and a behavioural test can significantly influence the value of a 'neurotoxic' dose derived from the rota-rod test, as showed by the present and Valzelli's (1973) results. Thus, the rota-rod test should be used with caution as the criterion for judging specificity of behavioural effects of drugs. Comparison of the changes in the behavioural activity in question (e.g., defensive upright posture) with other activities involving a similar type of movement but occurring in another behavioural context (e.g. exploratory rearing) in the same animal and setting (a procedure originally used by Silverman, 1965), seems to provide a more relevant as well as a more economic measure of selectivity of behavioural effects of drugs.

The incidence of 'adverse' effects seems to correlate rather with the ratio between LD_{50} and the lowest dose inhibiting timidity (LDIT): 'adverse' effects were less frequent or absent when the LD_{50} was more than ten times higher than LDIT. Diazepam had the largest 'safety margin'; its LDIT was 144 times smaller than its LD_{50} (Table 2).

Recording of non-timid activities in timid isolates can be helpful not only for assessment of the selectivity of timidity-reducing effects of drugs, as discussed above, but also for detection of aggression-evoking and sociability-increasing effects of drugs. Apart from barbitone, which significantly stimulated aggressive unrest in the present study, ethyl alcohol also evoked tail-rattling, aggressive unrest and attacks in timid mice (Kršiak, unpublished observation). The aggression-stimulating effects of drugs have so far been studied largely in aggressive animals (Kršiak, 1974). It may also be relevant to ascertain which drugs can facilitate aggressive behaviour in animals which usually do not respond aggressively to aversive stimulation. Timid singly-housed mice which do not usually attack their non-aggressive partners, yet appear to be potential attackers, seem to represent a convenient measure for detection of aggression-evoking activity of drugs.

Diazepam and barbitone increased sociability in timid mice whereas chlorpromazine and amphetamine did not. These findings are in agreement with effects of drugs on sociability in other species. Amylobarbitone increased social investigation and aggression in rats (Silverman, 1966a).

Chlordiazepoxide stimulated social sniffing in golden hamsters (Poole, 1973). On the other hand, both chlorpromazine and amphetamine increased escape from the partner in rats (Silverman, 1966b). Amphetamine decreased mutual grooming or sniffing in monkeys (Kjellberg & Randrup, 1973) and in rats (Schiørring & Randrup, 1971; Syme & Syme, 1973). Little is known as to what kind of psychotropic effect in man is predicted by increased sociability in animals. Nevertheless, the recording of sociable activities in timid mice might help to differentiate effects of sedatives relieving anxiety from those of neuroleptics.

The author wishes to thank Miss D. Vetíšková for her exceptional technical and observational assistance.

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(Received March 17, 1975.
Revised May 9, 1975.)

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THE PENETRATION OF DAPSONE, RIFAMPICIN, ISONIAZID AND PYRAZINAMIDE INTO PERIPHERAL NERVES

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1 Dapsone, rifampicin, isoniazid and pyrazinamide were shown to penetrate readily into the sciatic nerves of the dog and sheep.

2 These findings suggest that the continued persistence of viable drug-sensitive leprosy bacilli in the peripheral nerves of patients treated for long periods with either dapsone or rifampicin is not due to inadequate intraneural drug penetration.

Introduction

Dapsone (DDS) and rifampicin are two of the most important drugs used for the treatment of leprosy (Ellard, 1974). Daily treatment with 50 mg DDS reduces the number of viable *Mycobacterium leprae* in skin biopsies or nasal washings to less than 0.1% of their original total within about 3 months (Shepard, Levy & Fasal, 1968. 1972a) while a similar effect can be achieved by giving 600 mg rifampicin daily for as short a time as 1-3 weeks (Rees, Pearson & Waters, 1970; Shepard, Levy & Fasal, 1972b, 1974). Nevertheless, small numbers of viable *M. leprae* persist, often in preferred sites such as peripheral nerve and striated muscle (Pearson, Rees & Weddell, 1970) and, despite up to 10 years of continuous DDS treatment, these persisting bacilli can retain their sensitivity to DDS (Waters, Rees, McDougall & Weddell, 1974). Similar findings have also been encountered after up to 2 years daily treatment with rifampicin (Rees, 1975). These observations raised the possibility that both DDS and rifampicin might penetrate poorly into peripheral nerves.

To examine this hypothesis we investigated the penetration of DDS, rifampicin, isoniazid and pyrazinamide into the sciatic nerves of the dog and sheep. Isoniazid and pyrazinamide were chosen as suitable drugs for comparison since previous studies had indicated that they readily penetrate most body tissues. Thus both drugs are excellently absorbed in man, appear to be distributed throughout the total body water, are almost completely reabsorbed by the kidney, readily penetrate into the cerebrospinal fluid (CSF) and are highly effective against intracellular infections of *Mycobacterium tuberculosis* (Peters, Miller & Brown, 1965; Ellard, 1969; Gelber, Jacobsen & Levy, 1969; Weiner & Tinker, 1972; Ellard, Gammon & Tiitinen, 1973; Forgan-Smith, Ellard, Newton & Mitchison, 1973; Fox & Mitchison, 1975; Ellard & Gammon, unpublished observations). However, there appears to be very little knowledge concerning the penetration of these drugs, or indeed of other chemotherapeutic agents, into peripheral nerves.

Methods

The oral dosages of DDS, rifampicin, isoniazid and pyrazinamide given to the dog and the sheep are shown in Table 1. Each of the 4 drugs was given in the same animal on 2 occasions separated by an interval of 12 h so that their relative penetration into the nerves could be compared directly and so that moderately prolonged plasma concentrations would be maintained before removal of the nerves. Eight hours after the second dose, the animals were stunned with a humane killer, killed by exsanguination and the sciatic nerves immediately removed by meticulous exposure of the area concerned. After slitting the epineurium and taking care to avoid contact with other tissues or tissue fluids, segments of sciatic nerve were removed and placed directly in liquid nitrogen in which they were stored until analysis.

Plasma and ultrafiltrate (Toribara, Terepka & Dewey, 1957) concentrations of pyrazinamide were determined colorimetrically (Ellard, 1969); DDS, isoniazid and their potential metabolites monoacetyl-DDS and acetylisoniazid were determined fluorimetrically (Ellard & Gammon, 1969; Ellard, Gammon & Wallace, 1972), and rifampicin was assayed microbiologically by a plate-diffusion method with *Staphylococcus aureus* (Dickinson, Aber, Allen, Ellard & Mitchison, 1974).

Portions of the nerves weighing approximately 1 g were cut into small pieces, finely ground in a glass pestle and mortar with sand and then successively extracted by vigorously shaking on a vortex mixer with 2 x 10 ml chloroform/methanol (2 : 1 by volume) and 2 x 10 ml chloroform/methanol (1 : 2 by volume). The extracts were filtered and pooled.

For the determination of DDS and isoniazid, portions (10 ml) of the pooled extracts and of suitable aqueous standards diluted with chloroform/methanol (1 : 1) were evaporated to dryness on a rotary evaporator and the residues extracted by shaking with 2 ml 0.1N HCl plus 3 ml ethyl acetate. Two ml of the ethyl acetate extract was then diluted to 8 ml with ethyl acetate, extracted into 1.2N HCl and thence into ethyl acetate and the DDS determined. Isoniazid was determined by reacting portions of the 0.1N HCl extracts by the procedure used for its fluorimetric determination and correcting for the presence of fluorescent compounds derived from normal nerve by subtracting the fluorescence obtained when duplicate portions were pretreated with nitrous acid prior to reaction. For the microbiological determination of rifampicin, portions of the chloroform/methanol extracts of the nerve were taken through a solvent extraction procedure very similar to that described previously (Dickinson *et al.,* 1974) and the dried residues of the extracts dissolved in 0.3 ml portions of normal human serum. In the range of rifampicin concentrations assayed, the results obtained with aqueous standards and standards prepared by addition of rifampicin to extracts of normal sheep nerve did not differ significantly. Pyrazinamide was determined colorimetrically by drying a 10 ml portion of the chloroform/methanol dog nerve extract, extracting the residue with a mixture of 1 ml 0.1N H_2SO_4, 4 ml benzene/butan-1-ol (4 : 1 by volume) and 4 ml *n*-heptane, reacting 0.5 ml of the aqueous extract with alkaline nitroprusside and scanning the absorbance of the reaction product against an extract of normal (sheep) nerve treated in the same way.

Results

Preliminary experiments, in which single doses of the drugs were given to separate animals and plasma concentrations determined 1, 2, 4, 6 and 24 h later, revealed that in the dog the plasma half-life of isoniazid was about 4.5 h, pyrazinamide 5 h, rifampicin 10 h and DDS 18 hours. These values may be compared with estimates based on previously published data of 2.5, 5, 7-8 and 11 h, respectively (Peters, 1960; Cohn, 1969; Biggs, Gordon & Peters, 1971; Finkel, Pittillo & Mellett, 1971; Weiner & Tinker, 1972). Both DDS and isoniazid were acetylated by the sheep but neither compound was acetylated by the dog. Equivalent doses of DDS, rifampicin and isoniazid in the sheep resulted in lower but considerably more prolonged plasma concentrations than were attained in the dog. However, since measurable plasma concentrations of pyrazinamide were only achieved in the sheep for about 2 h after giving a 50 mg/kg dose of the drug, its penetration into sheep nerve could not be investigated. These preliminary studies, together with analyses on normal sheep nerve, confirmed the specificity of the methods used for the determination of all four drugs in both plasma and nerve. Furthermore, the fluorescence characteristics of the DDS and isoniazid extracts derived from the nerves of the treated animals were identical to those of aqueous standards and the rifampicin extracts failed to inhibit the growth of a rifampicin-resistant variant of the same *St. aureus* strain (Mitchison, Allen & Miller, 1970).

The concentrations of DDS, rifampicin,

Table 1 Concentrations of dapsone, rifampicin, isoniazid and pyrazinamide in the plasma, ultrafiltrate and sciatic nerve of the dog and sheep (μg/ml or μg/g)

Drug	*Species*	*Dose (mg/kg)*	*Plasma (hours after second dose)*					*Plasma ultrafiltrate at 8 h*	*Sciatic nerve at 8 h*
			0	*2*	*4*	*6*	*8*		
Dapsone	Dog	15	3.6	9.2	15.1	13.7	12.3	3.6	16.8
	Sheep	20	7.4	12.5	15.4	16.2	16.6	4.1	10.7
Rifampicin	Dog	20	7.2	11.3	31.0	28.3	24.6	1.5	4.4
	Sheep	50	8.3	9.5	10.9	11.7	13.3	1.1	1.3
Isoniazid	Dog	15	1.2	3.2	9.3	8.4	5.0	2.9	6.2
	Sheep	30	5.2	14.2	11.7	13.9	11.6	6.8	6.4
Pyrazinamide	Dog	50	7.7	47.7	35.7	29.2	20.8	20.8	20.0

isoniazid and pyrazinamide found in the plasma and nerve of the treated dog and sheep are summarized in Table 1. All four drugs readily penetrated into the sciatic nerve and in every case the concentrations found in the nerves equalled or exceeded the concomitant concentrations of the unbound drugs in the plasma. Since the vasculature of peripheral nerve is very slight, the contribution of any traces of blood present in the nerves after exsanguination to the estimated drug concentrations in the nerves would have been negligible. The highest nerve to ultrafiltrate drug ratios (3-5 fold) were encountered with the most lipid-soluble drug studied (DDS).

Discussion

The finding that pyrazinamide was not significantly bound by dog plasma proteins confirms previous evidence (Weiner & Tinker, 1972). By contrast isoniazid was approximately 40% protein-bound, DDS 70-75% protein-bound and rifampicin over 90% protein-bound by both dog and sheep plasma. The extent of protein binding of DDS by dog and sheep plasma was similar to that found by other workers for dog, human, rabbit, rat and mouse plasma (Biggs *et al.*, 1971). Several studies have demonstrated that rifampicin is highly bound to plasma proteins (Boman, 1973; Boman and Ringberger, 1974), but previous evidence for isoniazid protein-binding has been conflicting (Jenne, McDonald & Mendoza, 1961; Claunch, Castro & Barnes, 1963; Lloyd & Mitchison, 1964; Mattila & Takki, 1969). We found that whereas the acetylisoniazid formed *in vivo* by the sheep was not significantly protein-bound, the protein-binding of the monacetyl-DDS formed was virtually complete (unpublished observations). The extensive protein-binding of monoacetyl-DDS by plasma from other species has already been noted by other investigators (Biggs *et al.*, 1971).

The demonstration that DDS and rifampicin penetrated into sciatic nerve as readily as did isoniazid and pyrazinamide accords with other evidence concerning their tissue penetration. Thus DDS is well absorbed in man (Israili, Cucinell, Vaught, Davis, Lesser & Dayton, 1973) and evidence from the limited excretion of unchanged DDS when the pH of the urine is controlled to prevent the breakdown of the acid-labile DDS-*N*-glucuronide, and its relatively long half-life in man (Ellard & Gammon, 1969), suggests that some 97-98% of the protein-free drug is probably reabsorbed from the kidney. DDS has also been shown to penetrate into the muscle and brain of mice and rats (Francis, 1953; Murray, Gordon & Peters, 1974). Studies in the dog have demonstrated the excellent absorption of orally-administered rifampicin (Keberle, Schmid & Meyer-Brunot, 1968). Rifampicin also penetrates into human CSF (Forgan-Smith *et al.*, 1973), and leukocytes (Mandell, 1973), and whole body autoradiographic studies have shown it to enter the sciatic nerves of the mouse (Keberle *et al.*, 1968).

This experimental study, which has demonstrated the excellent penetration into the sciatic nerve of DDS, rifampicin and isoniazid in the dog and sheep and of pyrazinamide in the dog strongly suggests that they would also penetrate into human peripheral nerves. In the dog and sheep the concentrations of DDS found in nerve were actually 3-5 times higher than the concomitant concentrations of the unbound drug in the plasma and similar to the total plasma DDS concentrations. It must therefore be concluded that the persistence of small numbers of viable DDS-sensitive *M. leprae* in the peripheral nerves despite many years of treatment with DDS doses giving blood levels far in excess of its minimal inhibitory concentration against *M. leprae* (Ellard, Gammon, Rees & Waters, 1971; Waters *et al.*, 1974) cannot be due to inadequate tissue penetration of the drug.

It is suggested that unbound rifampicin readily penetrated into the sciatic nerves of both the dog and sheep but that the extensive protein-binding of the drug was responsible for the rifampicin concentrations in the nerve being only 10-20% of the total plasma concentrations of the drug. In view of the similar high degree of protein-binding of rifampicin by human plasma (Boman & Ringberger, 1974), analogous findings might also be expected in man. At present there is insufficient experimental evidence to indicate the possible influence of protein-binding on the antimycobacterial activity of rifampicin. If the antileprosy activity of rifampicin were determined solely by the concentrations of the unbound drug, one would expect it to be fully effective against intraneural *M. leprae.* If, however, its activity were a function of the total rifampicin concentrations achieved, reduced activity might be anticipated against intraneural bacilli. However, the results of a recent clinical trial (Rees & Waters, unpublished observations), in which the initial rates with which various daily dose sizes of rifampicin killed *M. leprae* were compared, suggest that even if the latter hypothesis were corect, a daily dose of 600 mg rifampicin should still exert a powerful antibacterial effect against intraneural *M. leprae.*

It must therefore be concluded that the persistence of viable drug-sensitive leprosy bacilli despite continued treatment with DDS or rifampicin cannot be due to inadequate tissue penetration of either drug. A more probable explanation is that a significant proportion of leprosy bacilli in tissue sites such as the nerves are dormant and as a consequence physiologically resistant to killing by either drug. DDS is in any case primarily a bacteriostatic drug, while it is known that rifampicin has very little bactericidal activity against non-growing *M. tuberculosis* (Dickinson, Jackett & Mitchison, 1972). This aspect of the chemotherapy of leprosy may therefore be similar to that encountered in the chemotherapy of tuberculosis where it is apparent that drugs that are highly bactericidal against acitvely growing *M. tuberculosis* are unable to kill dormant bacilli (Fox & Mitchison, 1975).

We should like to thank Professor J.W.T. Dickerson and D.A. Mitchison for their advice. Professor A.G.M. Weddell and Dr A.C. McDougall were supported by grants from the Medical Research Council and the British Leprosy Relief Association (LEPRA).

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(Received May 2, 1975.
Revised May 28, 1975.)

MEIOSIS: A PROSTAGLANDIN RESPONSE THAT IS NOT INHIBITED BY ASPIRIN

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Antidromic stimulation of the sensory nerve to the rabbit eye produces pupillary meiosis, supposedly by release of prostaglandins into the aqueous humour. The analogy between this phenomenon and neurogenic inflammation is drawn. It is shown that aspirin and indomethacin, known blockers of prostaglandin synthesis and release, fail to block the meiosis thus produced.

Introduction The role of the peripheral nervous system, and more particularly the axon reflex mechanism, in the inflammatory process has been studied by Janscó and co-workers (Janscó, 1966; Janscó, Janscó-Gábor & Szolcsanyi, 1967; Janscó, Janscó-Gábor & Szolcsanyi, 1968). They showed that the vasodilatation, increased capillary permeability and oedema typical of an inflamed tissue could be mediated solely by antidromic stimulation of peripheral sensory neurones such as can occur locally in the physiological axon reflex response. Further, using an unique pharmacological tool, they showed that blocking of the peripheral nerves greatly modified the inflammation produced locally by various noxious stimuli, which clearly implies that the nervous system does play a part in the inflammatory response under physiological conditions.

In the rabbit eye, the vascular inflammatory signs produced by antidromic stimulation of the sensory ophthalmic nerve were shown by Perkins (1957) to be manifest as increased permeability of ocular capillaries, increased intraocular pressure (equivalent to oedema), and pupillary meiosis, the latter being a smooth muscle effect probably mediated by capillary dilatation (Langworthy & Ortega, 1943). Similar effects in the eye are produced physiologically by a local axon reflex (Duke-Elder & Duke-Elder, 1932). There is good evidence to support the theory that the antidromically-induced effects in the eye are produced by release of prostaglandins into the aqueous humour (Ångård & Samuelsson, 1964; Ambache, Kavanagh & Whiting, 1965).

As there is now evidence linking prostaglandins with several inflammatory mechanisms (Ambache, 1966; Greaves, Sondergaard & McDonald-Gibson, 1971; Vane, 1971), and evidence implicating several anti-inflammatory drugs with synthesis and/or release of prostaglandins (Ferreira, Moncada & Vane, 1971, Smith & Willis, 1971; Vane, 1971) it was decided to ascertain whether aspirin (acetyl salicylic acid) or indomethacin would also modify the iris response to antidromic nerve stimulation.

Methods Six male albino rabbits (2.5-3.5 kg) were anaesthetized with urethane (1.5 g/kg, intravenously), blood pressure was monitored via a cannula in a common carotid artery, and body temperature maintained at a normal level with a water blanket. The animals were decerebrated to expose the trigeminal nerves and ganglia, and the dura overlying the ganglia was carefully removed. The ophthalmic nerves were cut on both sides peripheral to the ganglion.

After recovery from the ensuing meiosis (about 0.5 h), an intense unilateral pupillary constriction could be obtained by stroking the peripheral end of the cut ophthalmic nerve. The intensity and time course of recovery of this antidromically induced response was determined by measurement of the diameter of both pupils with a vernier caliber, at 5 min intervals.

The pupillary changes were followed in this manner for two control stimulations. Acetyl salicylic acid (200 mg/kg) in buffered solution (Cooke & Goulston, 1969) was then infused into the external jugular vein, and the mechanical stimulation of the nerve repeated at 30 and 60 min post infusion. Changes in pupillary diameter and the time course of recovery were noted as before. In 2 animals, up to 400 mg/kg of acetyl salicylic acid were given.

This protocol was also undertaken in an additional 4 rabbits substituting indomethacin (Merck, Sharpe & Dohme; 25 mg/kg) in buffered solution (Hucker, Zacchei, Cox, Brodie & Cantwell, 1966) for the acetyl salicylic acid infusate. A further 2 rabbits were pretreated with indomethacin (20 mg/kg, i.v.) at 24, 12 and 1 h before the decerebration and stimulation procedure.

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Results In every instance, mechanical stimulation, found by Perkins (1957) to give a more consistent response than electrical stimulation of the cut peripheral end of the ophthalmic nerve, resulted in an intense constriction of the pupil of the ipsilateral eye.

The pupil returned to its prestimulus size in 10-30 minutes. No change in the diameter of the contralateral pupil was observable during or after the stimulation in any animal.

Intravenous administration of acetyl salicylic acid solution had no appreciable effect in modifying the intensity or duration of any subsequent antidromically induced meiosis. This was true also for the rabbits which were acutely infused with indomethacin.

In the two animals which were pretreated with large doses of indomethacin, the response to antidromic stimulation of the cut trigeminal nerve was essentially the same as in untreated control rabbits, in intensity and duration.

Discussion It has been reported that meiosis can be produced by an axon reflex in the rabbit eye (Duke-Elder & Duke-Elder, 1932); hence antidromically mediated meiosis in the eye can be looked upon as the smooth muscle analogue of antidromically induced (or axon reflex produced) vascular inflammatory responses in other tissues. The results obtained in these experiments suggest that aspirin may not prevent all the nerve-dependent inflammatory signs in the eye. That is, if indeed it is the release of prostaglandins into the aqueous which initiates meiosis under these conditions, then this component of the inflammatory process is not totally inhibited by some known prostaglandin blockers. This observation is in good agreement with those of Neufeld, Jampol & Sears (1972), who found that, although aspirin will inhibit protein exudation into the aqueous humour induced by such insults to the eye as paracentesis or laser burns to the iris, the meiosis component of the inflammatory reaction is not prevented. It is particularly interesting in the light of Janscó's experiments (Janscó, 1966; Janscó *et al.,* 1967; Janscó *et al.,* 1968) with the neurogenic aspects of chemically induced inflammation, that Neufeld *et al.* (1972) also found that it is the inflammatory response to a chemical insult to the eye that is not blocked by aspirin. It is likely, therefore, that the nervous component is more significant in inflammation produced in response to a chemical stimulus, than to other types of stimulus.

The preliminary results from the indomethacin-treated rabbits must be assessed guardedly. There are no data on the accessibility of the aqueous to circulating indomethacin, although it is known to diffuse sparingly into the cerebrospinal fluid (Hucker *et al.,* 1966). Aspirin, on the other hand, appears to enter the aqueous in significant amounts (Neufeld *et al.,* 1972).

These data are interesting in that they suggest a model in which aspirin apparently does not totally prevent the release of prostaglandins which is subsequent to a given stimulus, although it is known to block prostaglandin release in several other preparations (Smith & Willis, 1971; Ferreira *et al.,* 1971).

An incidental observation of some interest was that, when constriction was produced in one pupil by stimulation of the cut ipsilateral ophthalmic nerve, no consensual changes in pupil diameter could be seen in the contralateral eye (with or without drug pretreatment), nor were blood pressure changes noted. This is not consistent with the hypothesis of Chiang & Thomas (1972) that the release (or injection, as in their experiments) of prostaglandins into the anterior chamber of one eye leads to consensual pressure changes in the contralateral eye, supposedly by way of prostaglandins released into the circulation. This may be explicable, however, on the grounds of type and dosage of drugs that were used in their injection experiments.

This research was financed by grant number MA-3638 to P.G. Dellow, and a Fellowship to T.S. Miles, from the Medical Research Council of Canada. Pure crystalline indomethacin was kindly donated by Dr W.D. Dorian, Merck, Sharpe & Dohme, Canada Ltd.

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(Accepted for publication April 1, 1973.
Resubmitted July 27, 1975.)

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Br. J. Pharmac. (1975), 55, 167-172

VASCULAR DOPAMINE RECEPTORS IN THE CANINE HINDLIMB

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1 Increases in femoral blood flow were produced by intra-arterial injections of dopamine (5-50 μg) in some but not all anaesthetized dogs studied, following treatment with the α-adrenoceptor antagonist, phentolamine.

2 The dilator effect of dopamine was not due to inhibition of adrenergic vasomotor tone as it was not affected by pharmacological procedures which completely abolished the activity of vasomotor nerves.

3 Blockade of vascular β-adrenoceptors using propranolol reduced the flow increases produced by dopamine much less than it did those produced by isoprenaline.

4 Responses to dopamine were significantly depressed by intra-arterial administration of ergometrine (0.5 mg). This dose of ergometrine did not reduce femoral dilator responses to acetylcholine, histamine, isoprenaline, bradykinin or 5-hydroxytryptamine.

5 It is concluded that the femoral vascular bed in the dog contains specific vasodilator receptors for dopamine. Ergometrine appears to be a selective antagonist of dopamine at these receptors.

Introduction

Several visceral vascular beds in the dog have been shown to contain dilator receptors for dopamine (DA) which are distinct from β-adrenoceptors (Eble, 1964; Goldberg, 1972). However, McNay & Goldberg (1966) reported that in the canine femoral vasculature, DA produced dilatation only in doses far larger than those which were effective in the visceral beds, and that this femoral response was mediated solely through activation of β-adrenoceptors. In the course of a continuing investigation of the vasodilator innervation of the canine hindlimb we have had occasion to re-examine the effects of DA in this vascular bed. This paper reports evidence for the presence of specific dopamine receptors, together with some further observations which reinforce our previously reported evidence for the usefulness of ergometrine as a selective antagonist of DA at such receptors (Bell, Conway & Lang, 1974).

Methods

Mongrel dogs of either sex weighing 10-20 kg were anaesthetized with α-chloralose (70 mg/kg i.v.) following induction with thiopentone sodium. The left femoral artery was exposed in the thigh and blood flow was recorded using a cuff-type electromagnetic flow probe. Systemic arterial pressure was recorded from a branch of the right femoral artery, and local injections of drugs were made into the aorta via a polythene catheter passed up the right femoral artery. α-Adrenoceptor blockade was maintained by hourly, or more frequent, injections of phentolamine mesylate (Regitine, Ciba) in a dose of 0.5 mg/kg intravenously. β-Adrenoceptor blockade was performed in some experiments by administration of propranolol (Inderal, ICI) in a dose of 0.05 mg/kg intravenously. In some experiments activation of vasomotor reflexes by dilator agonists was prevented by complete ganglion blockade with hexamethonium bromide (Sigma) 10 mg/kg intravenously or by pretreatment with guanethidine sulphate (Ismelin, Ciba) 5 mg/kg subcutaneously 18 and 2 hours before operation, instead of or as well as by α-adrenoceptor blockade.

Other drugs used were: acetylcholine chloride (Roche); atropine methonitrate (Sigma); bradykinin (Sandoz); dopamine hydrochloride (DA; BDH); ergometrine maleate (Burroughs Wellcome); histamine acid phosphate (David Bull); 5-hydroxytryptamine creatine phosphate (5HT; BDH); (±)-isoprenaline hydrochloride (Winthrop) and mepyramine maleate (May & Baker). Doses refer to the above salts. All agonists were freshly diluted with 0.9% w/v NaCl solution (saline) on the day of the experiment from frozen stock solutions, and injected as boluses of between 0.05 and 0.5 ml. Injection of 0.5 ml saline had no effect on femoral flow. Dilutions of DA and isoprenaline contained ascorbic acid 100 μg/ml.

Dilator responses were assessed as increases of blood flow (ml min^{-1} kg^{-1} body weight), and the significance of difference in responses before and after antagonist administration was examined using a paired Student's *t*-test. Comparison of the antagonism exerted by propranolol on responses to isoprenaline and dopamine was performed using two submaximal dose levels for each agonist, selected to give approximately equivalent dilator responses. After propranolol administration the doses of each agonist needed to produce responses approximately equal to those obtained under control conditions were obtained. Linear log dose-response curves were constructed for the control data in each experiment and used to calculate the exact dose-ratios necessary for equivalence of responses before and after propranolol. The significance of potency changes was assessed using the Behren-Fisher Statistic (Finney, 1952) on the assumption that no difference existed.

Results

Mean resting blood flow in the left femoral bed was 9.88 ± 1.63 ml min^{-1} kg^{-1} (mean ± s.e. mean). No deterioration in flow was seen over the 4-6 h period of the experiments.

Dilator agonists

The doses of dilator agonists used routinely were: acetylcholine (0.1-1 μg), histamine (5-50 μg), isoprenaline (0.05-0.5 μg), 5-hydroxytryptamine (0.1-50 μg), bradykinin (1-5 μg) and DA (5-50 μg). Acetylcholine, histamine, bradykinin and isoprenaline consistently caused increases in femoral blood flow and falls in systemic blood pressure at all dose levels tested. Responses to 5-hydroxytryptamine (5-HT) were more complex and will be discussed below (see *Specificity of ergometrine*). In 35 out of 77 dogs tested, DA caused femoral dilatation, while in the other animals no response was elicited by the range of doses used. The larger doses of dopamine (20-50 μg) consistently produced falls in blood pressure in those animals where a femoral dilator response was observed, but the smaller doses usually had no effect on blood pressure. Responses to acetylcholine were abolished by atropine (0.4 mg/kg i.v.) and those to histamine were reduced greatly or abolished by mepyramine (2 mg/kg i.a.). Neither of these antagonists affected responses to DA.

Rolè of ganglionic receptors in mediation of dopamine induced dilatation

Aortic administration of DA has been reported previously to produce femoral dilatation via inhibition of adrenergic vasomotor tone (Eble, 1964; Willems & Bogaert, 1973). This effect appeared to be mediated by an inhibitory action of DA on transmission through sympathetic ganglia. The possibility existed that in our experiments the α-adrenoceptor blockade with phentolamine was incomplete and allowed some residual vasomotor tone to persist. Other procedures were therefore adopted in some experiments to determine whether the DA-induced dilatations seen were due to action at the vascular muscle or at neuronal sites. In three dogs, the dilator effect of 20 μg DA was tested before and after administration of hexamethonium (10 mg/kg i.v.), which is sufficient to cause a sustained fall in blood pressure and abolition of vasomotor responses to preganglionic electrical stimulation. Responses to DA were unaffected by this drug treatment.

In a number of other animals pretreatment with guanethidine was utilized to abolish adrenergic vasomotor tone. The efficacy of this procedure was confirmed by the observation that subsequent administration of hexamethonium (10 mg/kg i.v.) did not cause any fall in blood pressure or increase in femoral blood flow. Dilator responses to DA of similar amplitudes to those seen in normal animals treated with phentolamine were observed in guanethidine-treated animals treated with phentolamine. We concluded that the femoral dilator response to DA which was being investigated was due to an action at sites on the vascular muscle rather than at vasomotor ganglionic synapses.

Role of β-adrenoceptors in the dopamine-induced dilatations

In order to test whether the femoral dilator effect of DA was due to activation of β-adrenoceptors as claimed by McNay & Goldberg (1966), a comparison was made of the antagonism of DA and of isoprenaline by the β-adrenoceptor antagonist propranolol (0.05-0.2 mg/kg i.v.). These doses of propranolol had no appreciable effect on resting femoral flow or on systemic blood

Table 1 Antagonistic effect of propranolol (0.1 mg/kg i.v.) on matched dilator responses to intra-aortic isoprenaline and dopamine on femoral blood flow in anaesthetized dogs treated with phentolamine (0.5 mg/kg i.v.)

	Isoprenaline				*Dopamine*			
Expt	*Response to isoprenaline, 0.05 μg (ml min^{-1} kg^{-1})*	*Propranolol dose-ratio*	*Response to isoprenaline, 0.1 μg (ml min^{-1} kg^{-1})*	*Propranolol dose-ratio*	*Response to dopamine, 10 μg (ml min^{-1} kg^{-1})*	*Propranolol dose-ratio*	*Response to dopamine, 20 μg (ml min^{-1} kg^{-1})*	*Propranolol dose-ratio*
1	1.25	8.0	1.38	2.5	1.18	1.1	1.59	1.1
2	1.0	50.0	1.07	2.0	1.0	1.5	1.21	1.7
3	1.09	5.0	1.87	5.0	1.21	1.4	1.45	1.0
4	0.68	3.6	1.13	5.6	0.49	0.8	0.83	1.1
5	0.85	10.0	2.20	20.0	0.85	1.0	1.70	0.7
6	0.30	3.1	0.46	4.2	0.23	0.5	0.30	0.4
7	0.33	4.0	0.85	5.0	0.80	3.3	0.95	2.9
Mean ± s.e. mean	0.79 ± 0.14	13.3**	1.28 ± 0.23	6.3*	0.82 ± 0.14	1.4	1.15 ± 0.19	1.3

* $P < 0.05$. ** $P < 0.01$.

Propranolol caused a considerable increase in the doses of isoprenaline necessary to match the control responses. The effect of propranolol on the potency of dopamine was much less pronounced. Results are expressed in terms of the initial increments in femoral flow produced by each agonist and the increase in dose necessary to produce responses of comparable magnitude following administration of propranolol, for seven separate experiments.

pressure. Propranolol appeared to depress responses to DA to a lesser and more variable extent than those to isoprenaline. A series of experiments was therefore performed to quantify the dose-ratio changes induced for submaximal doses of each agonist. Under control conditions isoprenaline (0.05 and 0.1 μg) gave dilator responses which approximated in size to those produced by 10 and 20 μg DA respectively (Table 1). In 7 experiments, administration of propranolol 0.1 mg/kg intravenously increased the doses of isoprenaline needed to produce responses of similar size by 13.3 and 6.3 fold respectively, while those of DA were increased by only 1.4 and 1.3 fold (Table 1). The control doses of isoprenaline and of DA used in this series of experiments produced only small decreases in arterial blood pressure. Thus isoprenaline (0.05 and 0.1 μg) lowered blood pressure by 2.1 ± 0 8 and 4.6 ± 0.8 mm Hg (mean ± s e mean) respectively and DA (10 μg and 20 μg) lowered blood pressure by 1.7 ± 0.6 and 4.0 ± 0.6 mm Hg (mean ± s.e. mean) respectively. Doses of each agonist needed to produce comparable flow responses after administration of propranolol also produced comparable depressor effects. Thus after propranolol isoprenaline (0 05 and 0.1 μg) lowered blood pressure by 1.3 ± 0.5 and 2.6 ± 0.8 mm Hg (mean ± s.e. mean) respectively and DA (10 μg and 20 μg) lowered blood pressure by 2.4 ± 0.5 and 3.9 ± 0.5 mm Hg (mean ± s.e. mean) respectively. We therefore felt justified in regarding the dilator activity of DA at least in the most part as due to activation of a receptor population distinct from β-adrenoceptors.

Effect of ergometrine on dopamine-induced dilatations

We have recently shown that ergometrine appears to act as a specific antagonist of DA at dopamine receptors in the canine renal vasculature (Bell *et al.*, 1974). It was therefore logical to test ergometrine as an antagonist of DA in the hind limb. Intra-aortic injection of ergometrine (0.5 mg) produced an immediate reduction in femoral blood flow which generally returned to the control resting level (±10%) within 1-2 minutes. Ergometrine also produced a slight increase in diastolic and a greater increase in systolic blood pressure, as described previously (Bell *et al.*, 1974). When femoral blood flow and blood pressure had stabilized following ergometrine injection, its effect on drug-induced dilator responses was investigated. In a series of 7 dogs, dose-response curves were obtained to DA, acetylcholine and histamine before and after intra-aortic injection of ergometrine. This procedure caused significant depression of responses to DA at all dose levels tested, but had no antagonistic effect on responses to the other agonists (Table 2).

Specificity of ergometrine

As an additional check of the proposal that DA

Table 2 Increments in femoral arterial blood flow (ml min^{-1} kg^{-1}) produced by intra-aortic injections of DA, acetylcholine, and histamine before and after intra-aortic administration of ergometrine (0.5 mg)

Agonist	*Controls*	*In presence of ergometrine (0.5 mg)*
Dopamine		
5 μg	0 52 ± 0.16 (5)	0.08 ± 0.08 (5)*
10 μg	0.96 ± 0.10 (6)	0.33 ± 0.11 (6)**
20 μg	1.19 ± 0 08 (6)	0.72 ± 0.08 (6)**
50 μg	2.70 ± 0 23 (7)	1.00 ± 0 26 (7)***
Acetylcholine		
0.1 μg	2 29 ± 0.74 (5)	2.27 ± 0.71 (5)
0.2 μg	2.46 ± 0.71 (3)	2.12 ± 0.71 (3)
0.5 μg	3.66 ± 0.93 (4)	4.58 ± 1.34 (4)
1.0 μg	4.16 ± 0.89 (7)	6.46 ± 0.91 (7)
Histamine		
2 μg	1.05 ± 0.31 (3)	1.36 ± 0.97 (3)
5 μg	2.67 ± 0.75 (4)	2.89 ± 1.17 (4)
20 μg	4.87 ± 1.27 (4)	5.01 ± 1.26 (4)
50 μg	4.91 ± 0.50 (3)	6.03 ± 1.56 (3)

* $P < 0.05$. ** $P < 0.005$. *** $P < 0.001$.
The number of experiments at each dose level are shown in parentheses. Values are means with s.e. mean.

was acting to produce femoral dilatation through receptors other than β-adrenoceptors, we examined the effect of ergometrine (0.5 mg intra-aortically) on responses to isoprenaline. In 6 experiments, there was slight potentiation of isoprenaline by ergometrine (Table 3). Although the absence of a depressant effect on responses to acetylcholine, isoprenaline and histamine indicated that ergometrine has no non-specific inhibitory effect on dilator responses in the dose used, its antagonism of DA could be explained through mechanisms other than blockade of a specific vascular DA receptor if one proposes that DA might act indirectly through local liberation of 5-HT or of a kinin and that ergometrine antagonizes the vasodilator effect of this substance. To exclude this possibility we tested the effect of ergometrine (0.5 mg intra-aortically) on femoral dilator responses to bradykinin and 5-HT. In 5 experiments, ergometrine caused potentiation of responses to 1-5 μg of bradykinin (Table 3). The predominant effect of 5-HT was to produce femoral constriction, and consistent increases in flow could be elicited only in some dogs tested, and then usually over only a narrow dose range which varied between individual animals. However, in 6 experiments of this type, ergometrine had no appreciable effect on the flow increases produced by 5-HT (Table 4).

Discussion

McNay & Goldberg (1966) reported that, in anaesthetized dogs, femoral dilator responses were elicited only with doses of DA ten or more times as high as those which produced renal vasodilatation. Furthermore, while the renal responses to DA were unaffected by β-adrenoceptor blockade, the femoral responses were abolished, suggesting the absence of specific DA receptors from the femoral bed.

Our results show a considerable divergence from those of McNay & Goldberg (1966). In about half of the dogs studied we could demonstrate femoral dilatation in response to doses of DA in the same range as are needed to produce renal vasodilatation (Eble, 1964; McNay & Goldberg, 1966; Bell *et al*, 1974). These responses were much less affected by β-adrenoceptor blockade than were those to isoprenaline, indicating that they were at least primarily not attributable to activation of β-adrenoceptors. However, our results do not preclude the possibility of appreciable β-adrenoceptor stimulation by DA in higher doses. Although Eble (1964) and Willems & Bogaert (1973) have demonstrated that DA can produce peripheral dilatation by inhibiting transmission through tonically active vasomotor ganglia, such a mechanism of action could not be evoked in our study since complete abolition of vasomotor tone by ganglion blockade or by guanethidine treatment did not prevent the dilator action of DA. We must conclude therefore that this effect of DA is due at least in the main to a direct action on the smooth muscle of the femoral vascular bed and not to activation of adrenoceptors. Support for a direct, dilator effect

Table 4 Increments in femoral arterial blood flow (ml min^{-1} kg^{-1}) produced by intra-aortic injections of 5-hydroxytryptamine (5-HT) before and after intra-aortic administration of ergometrine (0.5 mg)

Expt.	*5-HT (μg)*	*Control*	*After ergometrine*
1	1	0.96	0.96
2	5	1.10	1.08
	10	1.55	1.55
	40	2.17	1.86
3	40	1.17	1.10
4	10	0.34	0.34
5	1	0.45	0.70
6	1	0.25	0.25

Table 3 Increments in femoral arterial flow (ml min^{-1} kg^{-1}) produced by intra-aortic injections of isoprenaline and bradykinin before and after intra-aortic administration of ergometrine (0.5 mg)

	Control	*After ergometrine*
Isoprenaline		
0.1 μg	1.38 ± 0.37 (6)	1.63 ± 0.51 (6)
0.2 μg	2.21 ± 0.78 (5)	2.75 ± 0.66 (5)
0.5 μg	4.04 ± 1.06 (5)	4.25 ± 0.64 (5)
Bradykinin		
1 μg	1.25 ± 0.3 (4)	1.45 ± 0.48 (4)
2 μg	2.76 ± 0.65 (5)	2.79 ± 0.65 (5)
5 μg	2.89 ± 0.57 (3)	3.58 ± 1.01 (3)

of DA in the femoral bed has also come from the observations that DA-induced dilatation in the hindlimbs of conscious dogs is unaffected by α- and β-adrenoceptor blockade or by atropine (Higgins, Millard, Braunwald & Vatner, 1973).

Ergometrine has been reported to be an effective antagonist of DA in *Helix* ganglion (Walker, Woodruff, Glaizner, Sedden & Kerkut, 1968), and we have recently demonstrated that intra-arterial administration of ergometrine causes profound depression of dilator responses of DA in the dog renal vasculature without depressing responses to acetylcholine or histamine (Bell *et al.*, 1974). The present results have shown that intra-arterial injection of ergometrine produced profound depression of femoral dilator responses to DA while being devoid of antagonistic activity towards the dilator effect not only of acetylcholine and histamine but also of isoprenaline, bradykinin and 5-HT. They therefore strengthen further the position of ergometrine as a specific antagonist of DA in the mammalian vasculature.

The difference between our results and those of McNay & Goldberg (1966) may be due to several factors. McNay & Goldberg used animals in which the paw circulation was occluded, in order to study a more homogeneous vascular bed. Experiments in which we have examined femoral dilator responses with and without paw occlusion have indicated that an appreciable component of the dilator activity of DA is due to an effect within the vasculature of the paw. In view of the fact that we were unable to demonstrate a dilator response to DA in all animals tested, individual or strain differences between the receptor populations of different dogs may also be involved. A further possible factor is the use of barbiturate anaesthesia by McNay & Goldberg and of chloralose by ourselves. In this context, however, it may be noted that Eble (1964), using intact hindlimb preparations in barbiturate-anaesthetized dogs, observed femoral dilatation after α-adrenoceptor blockade in response to doses of DA similar to those necessary for the production of direct dilatation in the renal and mesenteric beds.

This work was supported by the Australian Kidney Foundation, the Life Insurance Medical Research Fund of Australia and New Zealand and by the Australian Research Grants Committee. E.L.C. was in receipt of a Commonwealth Postgraduate Scholarship. Regitine and guanethidine were kindly donated by Ciba-Geigy Australia Ltd.

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(Received January 6, 1975.
Revised April 29, 1975.
Re-revised June 4, 1975)

Br. J. Pharmac. (1975), 55, 173-179

THE EFFECTS OF HISTAMINE AND SOME HISTAMINE-LIKE AGONISTS ON BLOOD PRESSURE IN THE CAT

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1 The effects of histamine on blood pressure have been compared with the effects caused by four histamine-like agonists in anaesthetized cats.

2 It has been confirmed that the depressor responses to histamine involve both H_1- and H_2-receptors: depressor responses also follow the administration of selective H_1- and H_2-receptor agonists.

3 2-Methylhistamine, in doses up to 1×10^{-7} mol/kg, lowers blood pressure by interaction with H_1-receptors. Larger doses of 2-methylhistamine also lower blood pressure but this may involve H_2-receptors.

4 4-Methylhistamine, in doses up to 1×10^{-7} mol/kg, lowers blood pressure by interaction with H_2-receptors. Larger doses of 4-methylhistamine also lower blood pressure but this may involve H_1-receptors.

5 2-(2-Aminoethyl)pyridine and 2-(2-aminoethyl)thiazole both lower blood pressure by interaction with H_1-receptors only.

6 The potential value and limitations of these compounds as tools to investigate the cardiovascular effects of histamine are discussed.

Introduction

Many compounds have been described with histamine-like activity (for example Jones, 1966). Some of these histamine-like agonists interact selectively with either H_1- or H_2-receptors. Three agonists, 2-methylhistamine (Black, Duncan, Durant, Ganellin & Parsons, 1972; Durant, Ganellin & Parsons, 1975), 2-(2-aminoethyl)-pyridine and 2-(2-aminoethyl)thiazole (Lee & Jones, 1949; Grossman, Robertson & Rosiere, 1952; Durant *et al.*, in press), have been shown to interact selectively with H_1-receptors whereas 4-methylhistamine selectively interacts with H_2-receptors (Black *et al.*, 1972; Durant *et al.*, 1975).

This paper describes the effects of histamine and these selective histamine-like agonists on blood pressure in the cat. Histamine lowers blood pressure in the cat—a response which involves both H_1- and H_2-receptors (Black *et al.*, 1972; Owen & Parsons, 1974).

Methods

Experiments have been made in cats, of either sex, body weight 1.3-2.5 kg, anaesthetized by an intraperitoneal injection of sodium pentobarbitone (60 mg/kg). The trachea was cannulated. Blood pressure was measured from the right femoral artery, with a Statham P23A blood pressure transducer, and monitored on a Devices Electronic writer. Drugs were administered via cannulae in the right femoral vein or right brachial vein. Dose-response curves were constructed for histamine and histamine-like agonists by intravenous injections of increasing doses at intervals of 5 minutes.

Two series of experiments were done. The purpose of the first series was to determine the selectivity of each of the agonists for cardiovascular histamine receptors. In each experiment, one or two of the agonists were compared with histamine. Dose-response curves to histamine and the agonists were obtained in

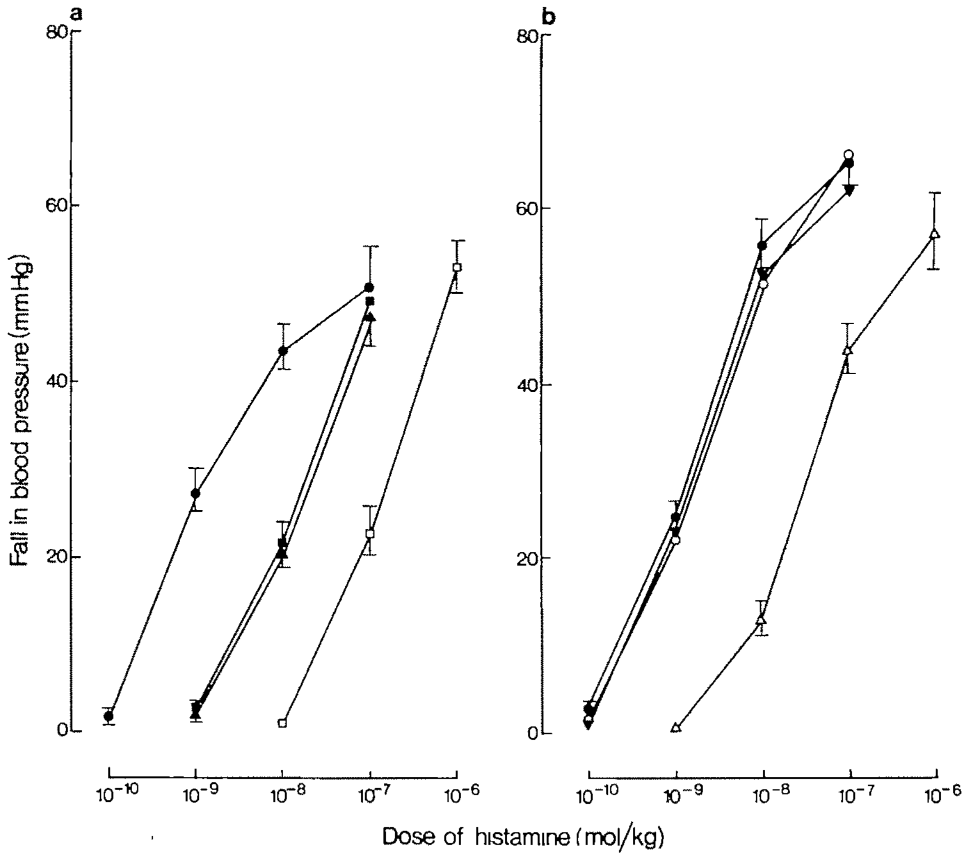

Figure 1 Anaesthetized cat blood pressure. The effect of mepyramine and metiamide on the depressor responses to histamine. (a) Effect of mepyramine alone and in combination with metiamide on the responses to histamine, $n = 11$. (●) Dose-response curve to histamine in the untreated cats; (■) after mepyramine 2.5×10^{-6} mol/kg; (▲) after mepyramine 2.5×10^{-5} mol/kg; (□) after treatment with mepyramine 2.5×10^{-5} mol/kg and metiamide 4×10^{-7} mol kg^{-1} min^{-1}. (b) Effect of metiamide alone and in combination with mepyramine on the histamine responses, $n = 11$. (●) Control dose-response curve; (○) after treatment with metiamide 4×10^{-7} mol kg^{-1} min^{-1}; (▼) after metiamide 2×10^{-6} mol kg^{-1} min^{-1}; (△) after metiamide 2×10^{-6} mol kg^{-1} min^{-1} and mepyramine 2.5×10^{-6} mol/kg. Vertical bars indicate s.e. mean.

untreated animals and then repeated after either H_1-receptor blockade with mepyramine or H_2-receptor blockade with metiamide. Mepyramine was given at two dose levels, 2.5×10^{-6} and 2.5×10^{-5} mol/kg. Displacement of the dose-response curves by mepyramine was evidence that the depressor responses were due to interaction with H_1-receptors. Metiamide was given by intravenous infusion at 4×10^{-7} mol kg^{-1} min^{-1} initially and then 2×10^{-6} mol kg^{-1} min^{-1}. Displacement of the dose-response curves by metiamide indicated interaction with H_2-receptors.

In the second series of experiments, made after determining the selectivity of the agonists for histamine receptors, the agonists were assayed for their depressor activity, relative to histamine under two conditions, firstly on H_1-receptors only during infusion of metiamide, 1×10^{-5} mol kg^{-1} min^{-1}, to block H_2-receptors and secondly on H_2-receptors only after injection of mepyramine, 2.5×10^{-5} mol/kg, to block H_1-receptors. Doses of histamine and the agonists were administered in random order. The results were treated by analysis of variance to provide estimates of potency.

Drugs used

Histamine acid phosphate (B.D.H.), mepyramine maleate (May & Baker), metiamide, 2-methylhistamine dihydrochloride, 4-methylhistamine

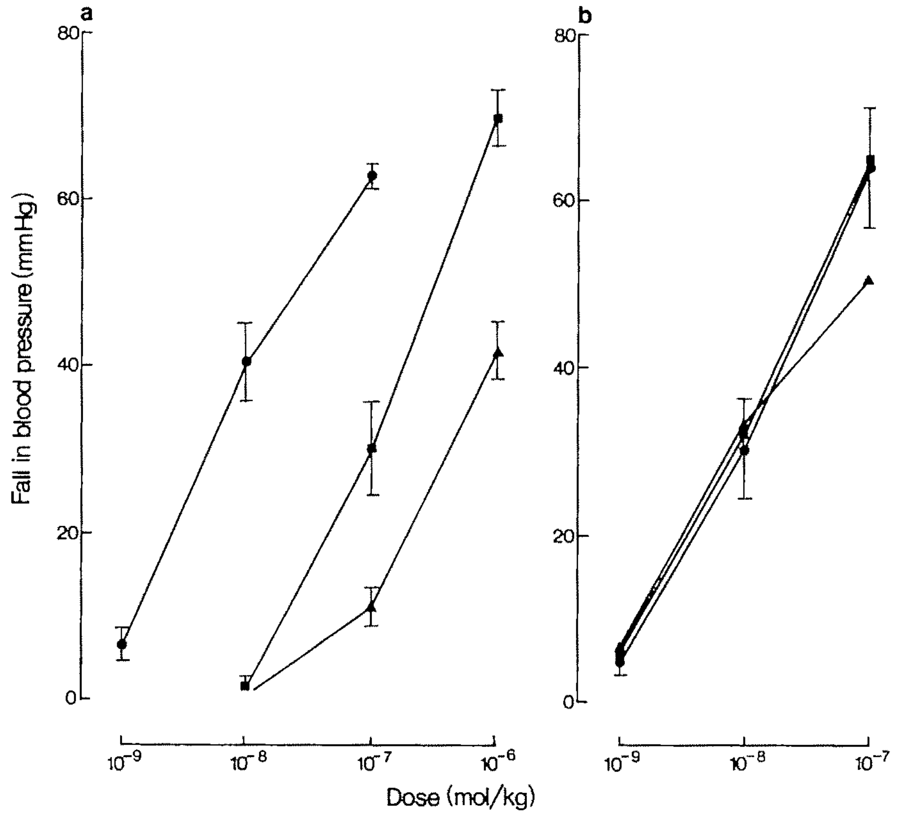

Figure 2 Anaesthetized cat blood pressure. The effect of two doses of mepyramine on the depressor responses to 2-methylhistamine (a), $n = 4$, and 4-methylhistamine (b), $n = 4$. (●) Dose-response curves before mepyramine; (■) after mepyramine, 2.5×10^{-6} mol/kg, (▲) after mepyramine, 2.5×10^{-5} mol/kg Mepyramine causes dose-dependent displacement of the 2-methylhistamine dose-response curve but had no effect on the responses to 4-methylhistamine. Vertical bars indicate s.e. mean.

dihydrochloride, 2-(2-aminoethyl)pyridine dihydrochloride and 2-(2-aminoethyl)thiazole dihydrochloride all synthesized by S.K. & F. Laboratories Limited, Welwyn Garden City.

Solutions of metiamide were prepared by dissolving the base in 0.1 N HCl. The pH of the solution was then raised to 7 with 0.1 N NaOH and the solution made up to volume with 0.9% NaCl solution. All other drugs were dissolved in 0.9% NaCl solution.

Results

Selectivity of the agonists for histamine-receptors

Histamine Both H_1- and H_2-receptors are involved in the depressor responses to histamine in the cat (Black *et al.*, 1972; Owen & Parsons, 1974). This has been confirmed in the present series of experiments. Histamine caused dose-dependent depressor responses over the dose-range 1×10^{-10} to 1×10^{-7} mol/kg. The histamine dose-response curve could be displaced to the right by mepyramine (2.5×10^{-6} mol/kg) but not by metiamide alone (up to 2×10^{-6} mol kg^{-1} min^{-1}). The displacement caused by mepyramine achieved a maximum at which point further displacement of the dose-response curve could only be achieved by infusions of metiamide (4×10^{-7} mol kg^{-1} min^{-1} and 2×10^{-6} mol kg^{-1}; Figure 1).

2-Methylhistamine 2-Methylhistamine caused dose-dependent depressor responses over the dose-range 1×10^{-9} to 1×10^{-7} mol/kg. Administration of mepyramine (2.5×10^{-6} mol/kg) caused a displacement of the dose-response curve to the right. This displacement was comparable to the displacement of histamine dose-response

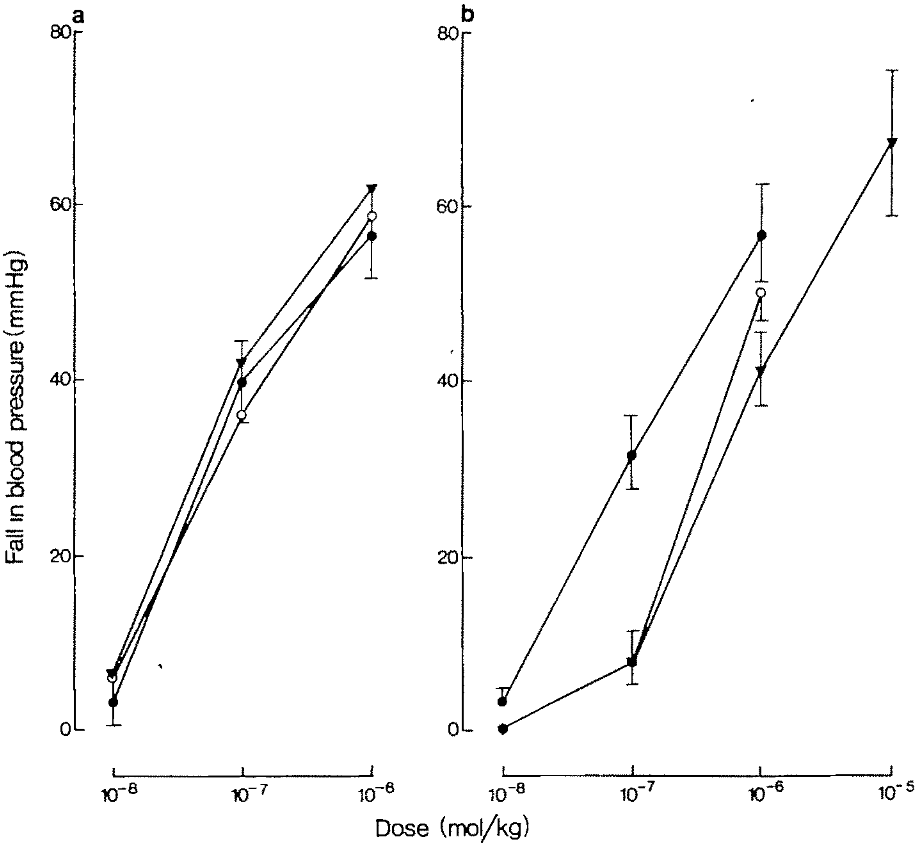

Figure 3 Anaesthetized cat blood pressure The effect of two doses of metiamide on the depressor responses to 2-methylhistamine (a), *n* = 4, and 4-methylhistamine (b), *n* = 4. (●) Dose-response curves before metiamide; (○) during infusion of metiamide 4×10^{-7} mol kg^{-1} min^{-1}; (▼) during infusion of metiamide, 2×10^{-6} mol kg^{-1} min^{-1}. Vertical bars indicate s.e mean.

curves by mepyramine (2.5×10^{-6} mol/kg). However, 2-methylhistamine differed from histamine because this dose of mepyramine did not cause maximal displacement of the dose-response curve. Increasing the dose of mepyramine to 2.5×10^{-5} mol/kg caused further displacement, to the right, of the 2-methylhistamine dose-response curve (see Figure 2). It was not possible to give a larger dose of mepyramine because of its toxic effects. It is likely that doses of 2-methylhistamine, greater than 1×10^{-7} mol/kg, administered in the presence of mepyramine (2.5×10^{-5} mol/kg), can interact with H_2-receptors, since administration of metiamide (4×10^{-7} mol kg^{-1} min^{-1}) caused a further displacement of the 2-methylhistamine dose-response curve.

Metiamide infusions (4×10^{-7} and 2×10^{-6} mol kg^{-1} min^{-1}) in the absence of mepyramine had no effect on the 2-methylhistamine dose-response curve (Figure 3).

These results indicate that the depressor responses to 2-methylhistamine are due to interaction with H_1-receptors. When large doses of 2-methylhistamine are given, in the presence of mepyramine, interaction with H_2-receptors can also occur.

4-Methylhistamine 4-Methylhistamine caused dose-dependent falls in blood pressure over the dose-range 1×10^{-8} to 1×10^{-6} mol/kg. The dose-response curve to 4-methylhistamine was not displaced by mepyramine (2.5×10^{-6} or 2.5×10^{-5} mol/kg; Figure 2). In contrast, metiamide (4×10^{-7} mol kg^{-1} min^{-1}) displaced the dose-response curve to the right. Increasing the dose of metiamide to 2×10^{-6} mol kg^{-1} min^{-1} caused no further displacement of the

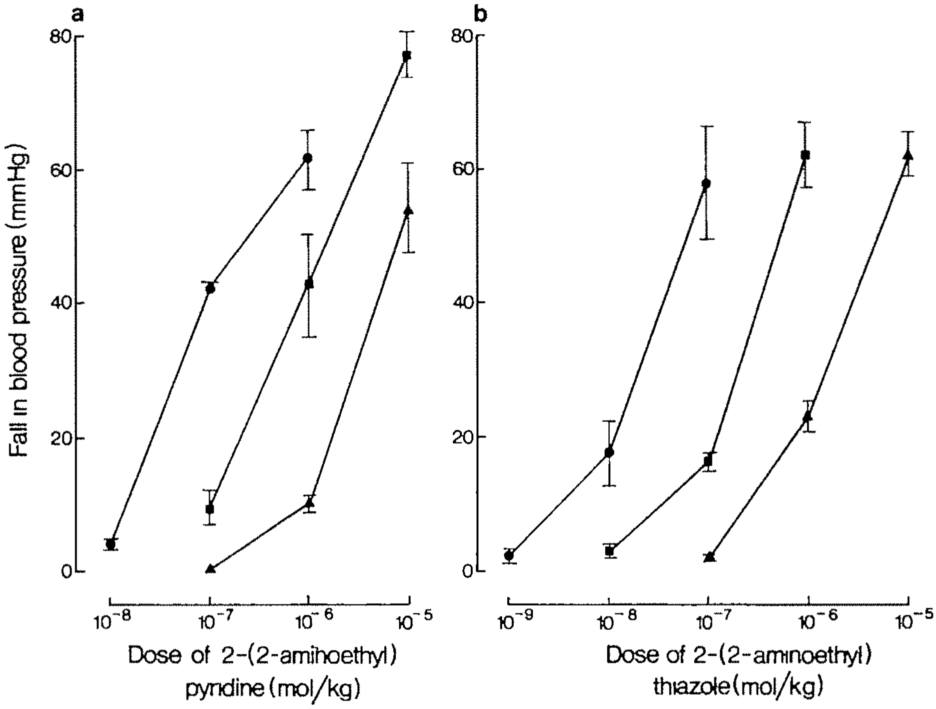

Figure 4 Anaesthetized cat blood pressure The effect of two doses of mepyramine on the depressor responses to 2-(2-aminoethyl)pyridine (a), $n = 4$ and 2-(2-aminoethyl)thiazole (b), $n = 4$. (●) Dose-response curves before mepyramine; (■) after mepyramine 2.5×10^{-6} mol/kg; (▲) after mepyramine, 2.5×10^{-5} mol/kg. Vertical bars indicate s.e mean

dose-response curve (Figure 3). However, administration of mepyramine (2.5×10^{-6} mol/kg) during the continuing infusion of metiamide caused further displacement of the dose-response curve. These results indicate that 4-methylhistamine in doses up to 1×10^{-7} mol/kg causes depressor responses by interaction with H_2-receptors; larger doses of 4-methylhistamine can interact with H_1-receptors.

2-(2-Aminoethyl)pyridine 2-(2-Aminoethyl) pyridine caused dose-dependent depressor responses over the dose-range 1×10^{-8} to 1×10^{-6} mol/kg. Mepyramine (2.5×10^{-6} mol/kg and 2.5×10^{-5} mol/kg) caused dose-dependent displacements of the dose-response curve to the right (Figure 4). Metiamide (up to 2×10^{-6} mol kg^{-1} min^{-1}) had no effect on the depressor responses to 2-(2-aminoethyl)pyridine either in control cats or when larger doses of 2-(2-aminoethyl)pyridine were given in the presence of mepyramine. These results indicate that the depressor responses to 2-(2-aminoethyl)pyridine are due entirely to interaction with H_1-receptors.

2-(2-Aminoethyl)thiazole 2-(2-Aminoethyl)thiazole caused dose-dependent falls in blood pressure over the dose-range 1×10^{-9} to 1×10^{-7} mol/kg. The dose-response curve was displaced to the right by mepyramine (2.5×10^{-6} mol/kg) and further to the right by increasing the dose of mepyramine 10-fold (Figure 4). Metiamide (up to 2×10^{-6} mol kg^{-1} min^{-1}) had no effect on dose-response curves obtained in untreated animals or in animals treated with mepyramine (2.5×10^{-5} mol/kg). These results indicate that the depressor responses to 2-(2-aminoethyl)thiazole are also due entirely to interaction with H_1-receptors.

Potency of the histamine-like agonists, relative to histamine

The potency of the agonists, relative to histamine was measured on both H_1- and H_2-receptors (Table 1).

Table 1 Relative potency of histamine and some histamine-like agonists on cardiovascular H_1- and H_2-receptors

	Relative Activity (95% confidence limits)	
Agonist	*H_1-receptors*	*H_2-receptors*
Histamine	100	100
2-methylhistamine	18.2 (13 7-24.0)	3.0 (1.6-5.4)
4-methylhistamine	0.13 (0.07-0.25)	23 7 (12.9-43.6)
2-(2-aminoethyl)pyridine	0.9 (0.7-1.2)	Inactive
2-(2-aminoethyl)thiazole	7.9 (6.0-10.5)	Inactive

Discussion

Histamine causes dose-dependent depressor responses which involve both H_1- and H_2-receptors (Black *et al.*, 1972; Owen & Parsons, 1974; Black, Owen & Parsons, 1975). This has been confirmed.

The present paper compares the effects of histamine and four histamine-like agonists on blood pressure in the cat. Prior to this study Black *et al.* (1972) had shown that 2-methylhistamine had greater potency on H_1-receptor systems than on H_2-receptor systems, whereas 4-methylhistamine was more effective on H_2-receptor systems than on H_1-receptor systems. The other two agonists, 2-(2-aminoethyl)pyridine and 2-(2-aminoethyl)thiazole, had both been shown to cause H_1-receptor effects only (Lee & Jones, 1949; Grossman *et al*, 1952, Durant *et al*, 1975).

Each of the agonists tested also caused dose-dependent depressor responses. Both 2-(2-aminoethyl)pyridine and 2-(2-aminoethyl)thiazole caused depressor responses by interaction with H_1-receptors and even when given at doses as high as 1×10^{-5} mol/kg there was no evidence of interaction with H_2-receptors.

2-Methylhistamine interacted selectively with H_1-receptors when given in doses up to 1×10^{-7} mol/kg causing large depressor responses in the cat. It was evident, however, that doses larger than 1×10^{-7} mol/kg could interact with H_2-receptors. When these larger doses were given, in the presence of mepyramine, the depressor responses were reduced by H_2-receptor blockade with metiamide. The selective H_2-receptor agonist activity of 4-methylhistamine could only be confirmed at doses up to 1×10^{-7} mol/kg. Although doses up to 1×10^{-7} mol/kg lowered blood pressure the responses were less than the maximum which could be obtained with 4-methylhistamine in untreated cats. Doses of 4-methylhistamine in excess of 1×10^{-7} mol/kg lowered blood pressure by interaction with both H_1- and H_2-receptors. In untreated cats mepyramine alone did not alter the responses to the smaller doses of 4-methylhistamine whereas metiamide alone did. The displacement of the dose-response curve by metiamide alone was not very large. The responses which persisted in the presence of metiamide were due to interaction with H_1-receptors and they were reduced or abolished by mepyramine.

When the selectivity of the agonists had been established they were assayed for H_1- and H_2-receptor activity relative to histamine. The measured potencies, relative to histamine, for 2-methylhistamine and 4-methylhistamine were very similar to the values reported by Black *et al.* (1972) on non-cardiovascular histamine receptors and by Flynn & Owen (1975) on vascular histamine receptors. The assay figures for 2-(2-aminoethyl)pyridine and 2-(2-aminoethyl)thiazole on blood pressure are also similar to the values determined by Flynn & Owen (1975) on vascular receptors. Although it is not possible to exclude other contributions to the depressor responses, dilatation of resistance vessels does appear to be a major cause of the depressor responses to bolus injection of histamine and histamine-like agonists.

The effects of histamine on the cardiovascular system are complex. Although both H_1- and H_2-receptors are involved in the gross blood pressure response to histamine it is possible that a detailed study of the component parts of the total response will reveal single receptor phenomena. Understanding of the parts of the total cardiovascular response has been greatly facilitated by the availability of both H_1- and H_2-receptor antagonists. In addition, the use of selective agonists will provide valuable backing to studies with the selective antagonists. These experiments in cats indicate that either 2-(2-aminoethyl)-pyridine or 2-(2-aminoethyl)thiazole can be used as selective H_1-receptor agonists in the cardiovascular system 2-Methylhistamine can also be used as a selective H_1-receptor agonist providing the doses used do not exceed 1×10^{-7} mol/kg since larger doses may also interact with H_2-receptors. 4-Methylhistamine may be used as a selective H_2-receptor agonist only at doses up to 1×10^{-7} mol/kg, which do not elicit maximum depressor responses in untreated cats.

I would like to thank Mrs Helen Farrington and Mrs Ann Wonnacott for their skilled assistance and Mr J.V. Smart for his statistical analysis of some of the results.

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(Received March 10, 1975)

Br. J. Pharmac. (1975), 55, 181-188

HISTAMINE RECEPTORS IN PERIPHERAL VASCULAR BEDS IN THE CAT

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1 The vasodilator activity of histamine has been studied in anaesthetized cats.

2 Histamine causes dose-dependent vasodilatation in the vasculature of the hind-limb and mesentery, perfused with blood at constant flow.

3 Experiments using the selective antagonists mepyramine and metiamide indicate the involvement of both H_1- and H_2-receptors in the vasodilator responses to histamine. Mepyramine (2.5×10^{-6} mol/kg), causes displacement of the histamine dose-response curve This displacement is maximum with a dose-ratio of about 10. Further dose-dependent displacement of the dose-response curve occurred after metiamide (4×10^{-7} mol kg^{-1} min^{-1} and 2×10^{-6} mol kg^{-1} min^{-1}), although these does of metiamide had no effect on histamine responses in the absence of mepyramine.

4 Vasodilator responses could also be elicited by the selective H_1-receptor agonists, 2-methylhistamine, 2-(2-aminoethyl)pyridine and 2-(2-aminoethyl)thiazole and the selective H_2-receptor agonist, 4-methylhistamine.

5 The selectivity of mepyramine and metiamide as histamine receptor antagonists was confirmed by their failure to reduce the vasodilator responses to acetylcholine, isoprenaline and bradykinin.

Introduction

In 1910 Dale & Laidlaw observed that in cats, intravenous injections of histamine produced dose-dependent falls in blood pressure due largely to dilatation of resistance vessels. The fall in blood pressure and vasodilatation are only partially blocked by H_1-receptor antagonists (Folkow, Haeger & Kahlson, 1948). It has now been shown that depressor responses to histamine involve both H_1- and H_2-receptors and can only be completely blocked by administration of both H_1- and H_2-receptor antagonists (Black, Duncan, Durant, Ganellin & Parsons, 1972; Owen & Parsons, 1974; Black, Owen & Parsons, 1975).

The present experiments were done to analyse the receptors involved in the vasodilator response to histamine in two major peripheral vascular beds of the cat. Part of this work was presented to the summer meeting of the British Pharmacological Society in Edinburgh, July 1974 (Flynn & Owen, 1974).

Methods

Cats of either sex, weighing between 1 5 and 2.8 kg were anaesthetized by intraperitoneal injection of sodium pentobarbitone (60 mg/kg). Supplementary injections of sodium pentobarbitone were given intravenously if necessary. The trachea was cannulated. The right brachial vein was cannulated for administration of mepyramine or metiamide.

Hind-limb perfusion

Systemic blood pressure was measured from a cannula in the right common carotid artery connected to a Statham P23A blood pressure transducer and recorded on a Devices M8 electronic recorder. The heart rate was measured from the blood pressure pulse using a ratemeter. The left hind-limb was acutely denervated by severing the sciatic nerve bundle in the popliteal cavity. The cat was given heparin 1000 i.u./kg. The right femoral artery was cannulated and blood pumped through silicone rubber tubing by a Watson-Marlow flow inducer at constant pulsatile flow into the left femoral artery. A small segment of thick rubber tubing was inserted into the external perfusion circuit before the pump for injection of drugs. The perfusion pressure was measured by a blood pressure transducer from a side arm in the perfusion circuit between the pump and the perfused hind-limb. At the start of the experiment the rate of flow was adjusted so that the perfusion pressure was approximately equal to systemic blood pressure and was kept constant at this rate for the duration of the experiment.

Superior mesenteric perfusion

Systemic blood pressure was recorded from the right femoral artery. The inferior mesenteric artery was tied off. The superior mesenteric artery was cleared of connective tissue and the sympathetic nerves around it cut. Blood was taken from the right common carotid artery and pumped through the superior mesenteric artery at constant flow. Perfusion pressure was measured between the pump and vascular bed. As in the hind-limb preparation, drugs were injected into the perfusion system just before the pump.

In both vascular beds intra-arterial injections of vasodilators caused dose-dependent decreases in perfusion pressure, indicating decreases in vascular resistance. When responses, expressed as decreases in perfusion pressure in mmHg, were plotted against $\log_{10}$ dose there was an increase in slope of the linear part of repeated dose-response curves during the course of some experiments. This increase in slope was correlated with a slow increase in perfusion pressure that occurred during the course of these experiments. Expressing the vasodilatation as percentage decrease in perfusion pressure permitted better reproducibility of dose-response curves, eliminating much of the increase in slope.

The effects of mepyramine and metiamide on histamine vasodilatation were determined in both vascular beds. Studies with the histamine-like agonists and non-histamine vasodilators were restricted to the hind-limb vasculature.

In each experiment the doses of agonists were given in random order. Analysis of variance was used to estimate the statistical significance of the results, tests being made for linearity, curvature and parallelism of the dose-response curves. Displacements of the dose-response curves caused by the antagonists were estimated by calculating the potency of the agonist in the presence of the antagonist compared with the potency in the untreated animal, using the data from the analysis of variance.

The slope used in estimating displacements of the dose-response curves was the mean slope of the dose-response curves in that group of experiments.

Drugs used

Histamine acid phosphate (B.D.H.), 2-methylhistamine dihydrochloride, a histamine-like agonist selective for H_1-receptors (Black *et al*, 1972), 4-methylhistamine dihydrochloride, a histamine-like agonist selective for H_2-receptors (Black *et al.*, 1972), 2-(2-aminoethyl)pyridine dihydrochloride and 2-(2-aminoethyl)thiazole dihydrochloride, both histamine-like agonists selective for H_1-receptors (Lee & Jones, 1949, Grossman, Robertson & Rosiere, 1952; Durant, Ganellin & Parsons, to be published), metiamide, mepyramine maleate (May & Baker), acetycholine chloride (B.D.H.), (−)-isoprenaline hydrochloride (Abbott) and synthetic bradykinin (Sandoz).

Solutions of metiamide were prepared by dissolving the base in a small volume of 0.1 N HCl, neutralizing the solution with 0.1 N NaOH and making up to the required volume with 0.9% NaCl solution.

Histamine, 2-methylhistamine, 4-methylhistamine, 2-(2-aminoethyl)pyridine, 2-(2-aminoethyl)thiazole, acetylcholine, isoprenaline and bradykinin were dissolved in 0.9% NaCl solution. These vasodilators were given by intra-arterial injection into the external perfusion circuit before the pump in volumes of 10-20 µl.

Mepyramine was given by slow intravenous injection. Because of its shorter duration of action, metiamide was given continuous intravenous injection at a flow rate of 0.2 ml/minute. Infusions of metiamide were made for 30 min before and continued during the injection of vasodilators.

Results

Hind-limb vascular bed

Intra-arterial injection of histamine over the dose-range 1×10^{-12} to 1×10^{-7} mol/kg caused dose-dependent decreases in perfusion pressure. Doses greater than 1×10^{-8} mol/kg passed into the systemic circulation and lowered blood pressure.

Administration of the histamine H_1-receptor antagonist, mepyramine (2.5×10^{-6} mol/kg), caused a displacement of the histamine dose-response curve to the right with a dose-ratio of 10.10 (8.7 to 11.7, 95% confidence limits, 4 experiments) Additional doses of mepyramine up to a total cumulative dose of 2.5×10^{-5} mol/kg caused no further displacement of the histamine dose-response curve.

Infusions of the histamine H_2-receptor antagonist metiamide, up to doses of 2×10^{-6} mol kg^{-1} min^{-1}, did not significantly alter the histamine dose-response curve.

Administration of both mepyramine and metiamide together caused a large displacement of the histamine dose-response curve. After maximum displacement of the dose-response curve by H_1-receptor blockade, administration of

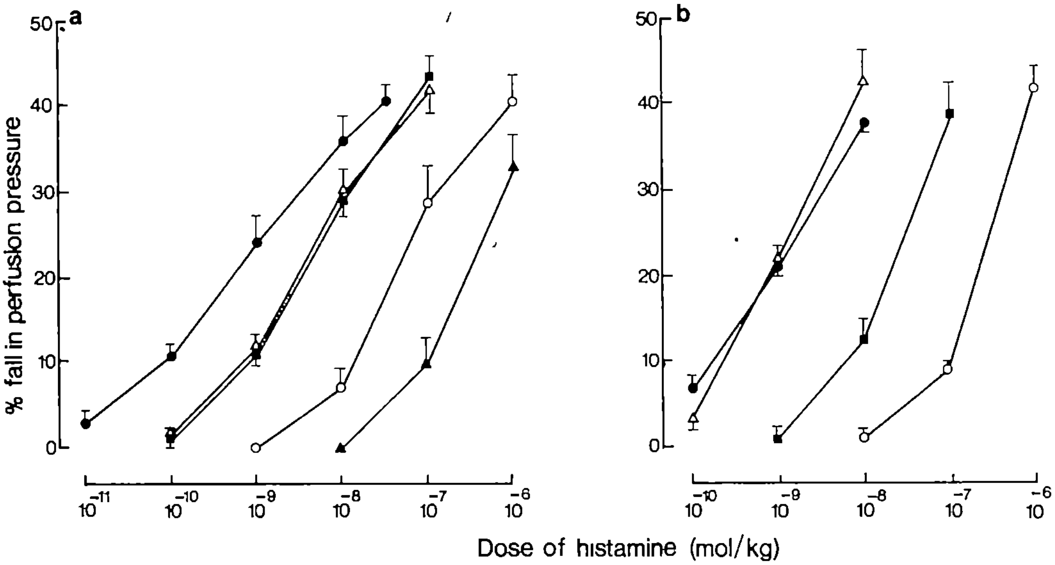

Figure 1 Anaesthetized cats. Vasodilator response to histamine in the hind-limb perfused with blood at constant flow rates. The effects of mepyramine and metiamide on the responses to histamine. (a) Dose-response curve to histamine in untreated cats (●), (△) after mepyramine 2.5 x 10^{-5} mol/kg; (■) after mepyramine 5 x 10^{-5} mol/kg, (○) after mepyramine 5 x 10^{-5} mol/kg plus metiamide 4 x 10^{-7} mol kg^{-1} min^{-1}; (▲) after mepyramine 5 x 10^{-5} mol kg^{-1} min^{-1} plus metiamide 2 x 10^{-6} mol kg^{-1} min^{-1}. (b) Dose-response curve to histamine in untreated cats (●); (△) after metiamide 2 x 10^{-6} mol kg^{-1} min^{-1}, (■) after metiamide 2 x 10^{-6} mol ,kg^{-1} min^{-1} plus mepyramine 2.5 x 10^{-6} mol/kg, (○) after metiamide 2 x 10^{-6} mol kg^{-1} min^{-1} plus mepyramine 2.5 x 10^{-5} mol/kg. Vertical bars indicate s.e. mean.

metiamide caused dose-dependent displacements of the histamine dose-response curve to the right.

Relative to the curve in the presence of mepyramine 5 x 10^{-5} mol/kg, metiamide caused shifts with dose-ratios of 13.8 (6.9 to 27.8, 95% confidence limits, 4 experiments) at an infusion rate of 4 x 10^{-7} mol kg^{-1} min^{-1} and 51.6 (22.2 to 119.8, 95% confidence limits, 3 experiments) at an infusion rate of 2 x 10^{-6}mol kg^{-1} min^{-1}. These changes are illustrated in Figure 1a.

Administration of mepyramine during the infusion of metiamide (2 x 10^{-6} mol kg^{-1} min^{-1}) caused dose-dependent displacement of the histamine dose-response curve to the right, with dose-ratios of 10.0 (8.4 to 11.8, 95% confidence limits, 6 experiments) and 154 (130 to 183, 95% confidence limits, 6 experiments) at 2.5 x 10^{-6} mol/kg and 2.5 x 10^{-5}mol/kg mepyramine respectively. These changes are illustrated in Figure 1b.

Superior mesenteric vascular bed

Intra-arterial injection of histamine over the range 1 x 10^{-11} to 1 x 10^{-8} mol/kg caused dose-dependent decreases in perfusion pressure. Doses greater than 1 x 10^{-8} mol/kg contracted the gut, which interfered with the vasodilatation responses, and some of the histamine passed into the systemic circulation causing depressor responses. The vasodilator responses were linear over the range 1 x 10^{-11} to 1 x 10^{-9} mol/kg.

Administration of mepyramine (2.5 x 10^{-5} mol/kg), caused a displacement of the histamine dose-response curve to the right with a dose-ratio of 16.3 (6.9 to 38.3, 95% confidence limits, 4 experiments). Administration of a further dose of 2.5 x 10^{-5} mol/kg mepyramine, making a cumulative dose of 5 x 10^{-5} mol/kg, had no further effect on the dose-response curve. Infusions of metiamide, up to a dose of 2 x 10^{-6} mol kg^{-1} min^{-1}, had no significant effect on the histamine dose-response curve.

Large displacements of the histamine dose-response curve could be obtained with combinations of the H_1- and H_2-receptor antagonists. Having obtained a maximal displacement of the histamine dose-response curve with mepyramine, 5 x 10^{-5} mol/kg, the dose-response curve could be displaced further to the right by metiamide 2 x 10^{-6} mol kg^{-1} min^{-1}, with a dose-ratio of 114 (49 to 264, 95% confidence

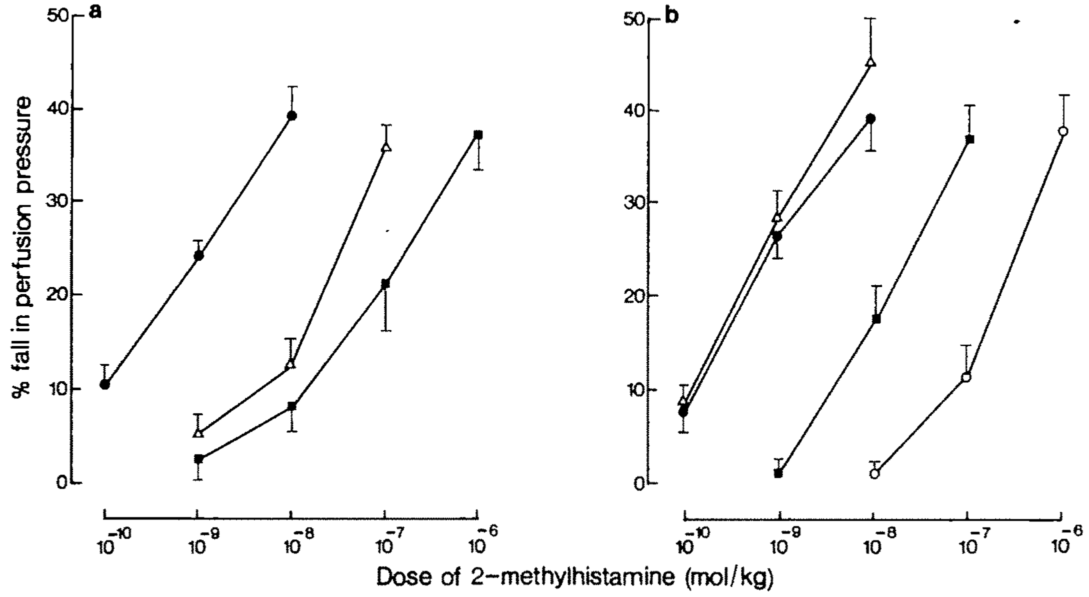

Figure 2 Anaesthetized cats. The effects of mepyramine and metiamide on the vasodilator responses to 2-methylhistamine. (a) Dose-response curve to 2-methylhistamine in untreated cats (●), (△) after mepyramine 2.5 x 10^{-5} mol/kg, (■) after mepyramine 2.5 x 10^{-5} mol/kg; plus metiamide 2 x 10^{-6} mol kg^{-1} min^{-1}. (b) Dose-response curve to 2-methylhistamine in untreated cats (●); (△) after metiamide 2 x 10^{-6} mol kg^{-1} min^{-1}; (■) after metiamide 2 x 10^{-6} mol kg^{-1} min^{-1} plus mepyramine 2.5 x 10^{-6} mol/kg; (○) after metiamide 2 x 10^{-6} mol kg^{-1} min^{-1} plus mepyramine 2.5 x 10^{-5} mol/kg. Vertical bars indicate s.e. mean.

limits, 4 experiments) relative to the dose-response curve in the presence of mepyramine.

During infusion of metiamide, 2 x 10^{-6} mol kg^{-1} min^{-1}, mepyramine caused dose-dependent displacements of the histamine dose-response curve to the right with a dose-ratio of 62.1 (41.5 to 92.7, 95% confidence limits, 4 experiments) at 2.5 x 10^{-6} mol/kg and a dose ratio of 1,333 (894 to 1989, 95% confidence limits, 4 experiments) at 2.5 x 10^{-5} mol/kg.

Effect of histamine-like agonists on hind-limb vasculature

2-Methylhistamine caused dose-dependent vasodilator responses over the dose-range 1 x 10^{-10} to 1 x 10^{-8} mol/kg. Over this dose-range the dose-response curve was linear. Administration of mepyramine, 2.5 x 10^{-5} mol/kg, caused a parallel displacement of the 2-methylhistamine dose-response curve to the right (Figure 2a) with a dose-ratio of 29.2 (16.1 to 52.9, 95% confidence limits, 4 experiments), whereas metiamide alone up to 2 x 10^{-6} mol kg^{-1} min^{-1} had no effect on the 2-methylhistamine dose-response curve. When metiamide (2 x 10^{-6} mol kg^{-1} min^{-1}) was administered in the presence of mepyramine (2.5 x 10^{-5} mol/kg) it had no effect on the responses to the smaller dose of 2-methylhistamine, up to 1 x 10^{-8} mol/kg, but did reduce the responses to larger doses of 2-methylhistamine (Figure 2b).

In the untreated cat, vasodilator responses to 4-methylhistamine were linear over the range 1 x 10^{-10} to 1 x 10^{-8} mol/kg. In contrast to the effect of mepyramine on the histamine dose-response curve, administration of mepyramine 2.5 x 10^{-5} mol/kg, had no effect on the 4-methylhistamine dose-response curve (Figure 3a).

Infusion of metiamide (2 x 10^{-6} mol kg^{-1} min^{-1}) alone caused a displacement of the 4-methylhistamine dose-response curve to the right (Figure 3b) with a dose-ratio of 11.6 (6.9 to 19.4, 95% confidence limits, 4 experiments). When displaced to the right by metiamide (2 x 10^{-6} mol kg^{-1} min^{-1}) the 4-methylhistamine dose-response curve was further shifted to the right by administration of mepyramine, 2.5 x 10^{-6} mol/kg, with a dose-ratio relative to the position of the dose-response curve in the presence of metiamide, of 12.3 (7.3 to 20.6, 95% confidence limits, 4 experiments).

In the untreated cat, responses to

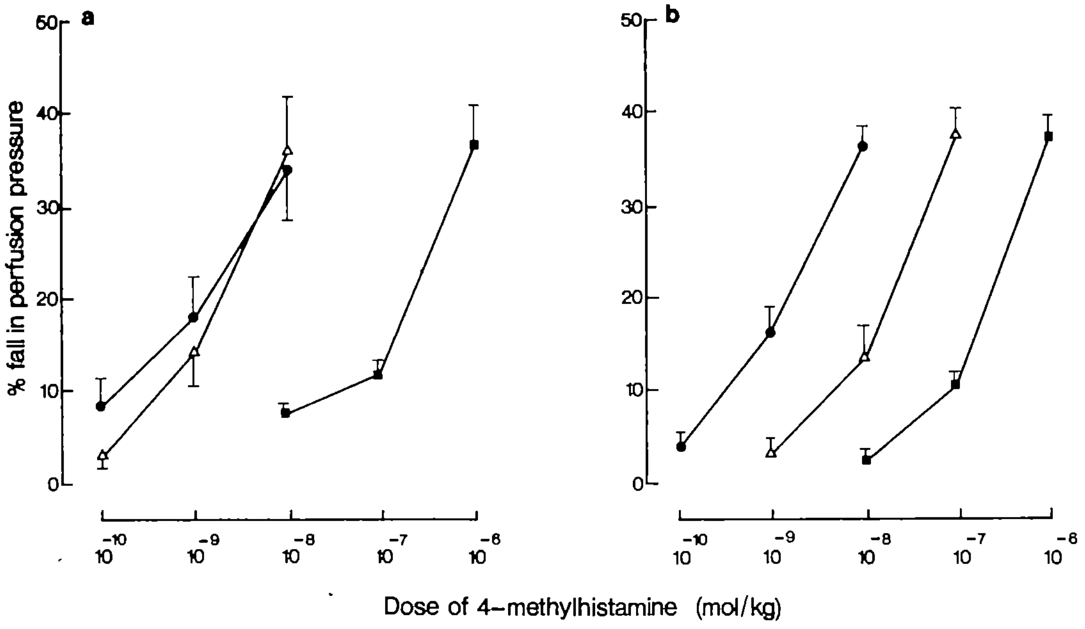

Figure 3 Anaesthetized cat. Effect of mepyramine and metiamide on the vasodilator responses to 4-methylhistamine. (a) Dose-response curve to 4-methylhistamine in untreated cats (●); (△) after mepyramine 2.5×10^{-5} mol/kg; (■) after mepyramine 2.5×10^{-5} mol/kg plus metiamide 2×10^{-6} mol kg^{-1} min^{-1}. (b) Dose-response curve to 4-methylhistamine in untreated cats (●); (△) after metiamide 2×10^{-6} mol kg^{-1} min^{-1}; (■) after metiamide 2×10^{-6} mol kg^{-1} min^{-1}; plus mepyramine 2.5×10^{-6} mol/kg. Vertical bars indicate s.e. mean.

2-(2-aminoethyl)thiazole were linear over the range 1×10^{-10} to 1×10^{-8} mol/kg. Administration of mepyramine caused dose-dependent displacements of the 2-(2-aminoethyl)thiazole dose-response curve to the right, with dose-ratios of 14.3 (10.5 to 19.5, 95% confidence limits) and 44.0 (32.6 to 60.2, 95% confidence limits, 6 experiments), at 2.5×10^{-6} and 2.5×10^{-5} mol/kg respectively. Subsequent administration of metiamide (4×10^{-7} mol kg^{-1} min^{-1}) did not significantly affect the dose-response curve.

In the untreated cat, responses to 2-(2-aminoethyl)pyridine were linear over the range 1×10^{-9} to 1×10^{-7} mol/kg. Administration of mepyramine caused dose-dependent displacements of the 2-(2-aminoethyl)pyridine dose-response curve to the right with dose-ratios of 15.2 (10.5 to 22.0, 95% confidence limits, 6 experiments), and 39.0 (26.6 to 57.1, 95% confidence limits, 6 experiments) at 2.5×10^{-6} and 2.5×10^{-5} mol/kg respectively. Subsequent administration of metiamide (4×10^{-7} mol kg^{-1} min^{-1}) had no effect on the dose-response curve.

Potency of the histamine-like agonists relative to histamine

Each of the agonists was assayed for potency, relative to histamine on vascular H_1- and/or H_2-receptors. The values are shown in Table 1. The assay for H_1-receptor activity was made in the presence of metiamide (2×10^{-6} mol kg^{-1} min^{-1}) to ensure blockade of H_2-receptors; the assay for H_2-receptor activity was made in the presence of mepyramine (2.5×10^{-5} mol/kg) to ensure H_1-receptor blockade.

Specificity of the histamine antagonists

To establish the specificity of mepyramine and metiamide as histamine receptor antagonists, their effects on vasodilator responses to non-histamine-like vasodilators was studied. One dose of bradykinin, acetylcholine, isoprenaline and histamine was determined to give approximately equal sub-maximal responses. The doses used were 3.0×10^{-8} mol/kg acetylcholine, 6.0×10^{-9} mol/kg isoprenaline, 4×10^{-9} mol/kg bradykinin

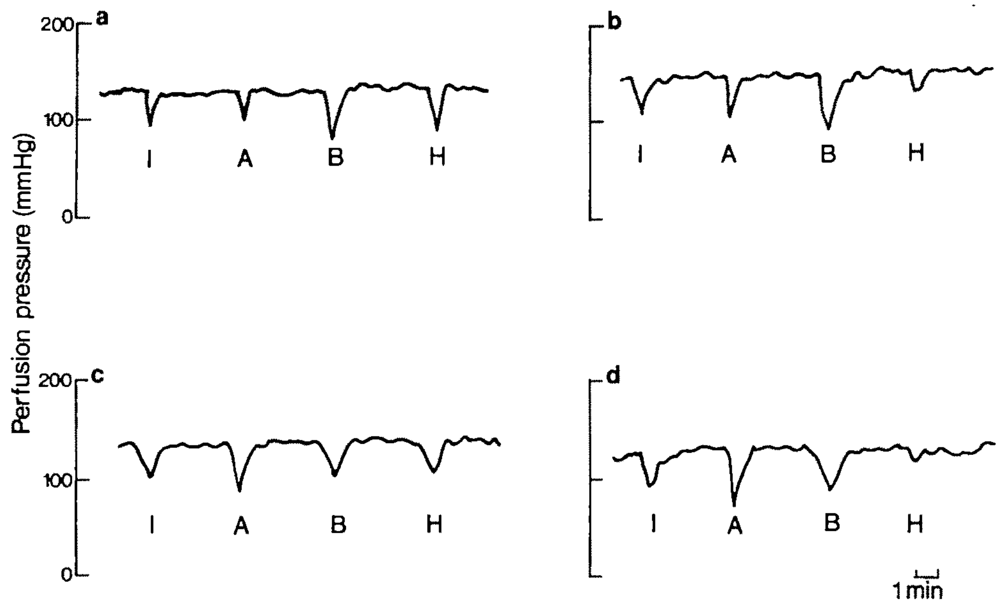

Figure 4 Anaesthetized cats. Effect of mepyramine and metiamide on the vasodilator responses to isoprenaline 6.0×10^{-9} mol/kg at I, acetylcholine 2.8×10^{-8} mol/kg at A, bradykinin 4.0×10^{-9} mol/kg at B and histamine 1×10^{-7} mol/kg at H. (a) Responses in untreated cats. (b) Responses after mepyramine 2.5×10^{-5} mol/kg which reduced the response to histamine but had no effect on the other responses. (c) Responses are shown during infusion of metiamide, 2×10^{-6} mol kg^{-1} min^{-1} which had no effect on the responses. (d) Responses after treatment with mepyramine 2.5×10^{-5} mol/kg plus metiamide 2×10^{-6} mol kg^{-1} min^{-1} which abolished the responses to histamine but had no effect on the other vasodilator responses.

and 1.0×10^{-7} mol/kg histamine. Each dose was given twice and the eight doses given in random order. The eight doses were repeated in the presence of mepyramine (2.5×10^{-5} mol/kg) alone, metiamide (2×10^{-6} mol kg^{-1} min^{-1}) alone and a combination of mepyramine (2.5×10^{-5} mol/kg) and metiamide (2×10^{-6} mol kg^{-1} min^{-1}). The results are shown in Figure 4.

The responses to histamine were reduced by mepyramine alone and by the combination of mepyramine and metiamide. The responses to the other agonists were not significantly reduced by either mepyramine alone, metiamide alone or by the combination of mepyramine and metiamide.

Discussion

The purpose of these experiments was to determine the histamine receptors involved in histamine-induced vasodilatation. Experiments were made in two major peripheral vascular beds and essentially similar results were obtained.

Prior to this, Folkow *et al.* (1948) had shown that whereas the fall in systemic blood pressure and vasodilatation caused by small doses of histamine could be reduced or abolished by H_1-receptor antagonists, the responses to larger doses of histamine were refractory to H_1-receptor antagonists. These findings prompted Folkow *et*

Table 1 Potency of histamine and some histamine-like agonists on vascular H_1- and H_2-receptors

	Potency (95% confidence limits)	
Agonist	*H_1-receptors*	*H_2-receptors*
Histamine	100	100
2-Methylhistamine	15.0 (9.8-23.0)	2.3 (1.4-3.8)
4-Methylhistamine	0.5 (0.3- 0.8)	37.4 (21.4-65.6)
2-(2-Aminoethyl)pyridine	2.3 (1.5- 3.4)	Inactive
2-(2-Aminoethyl)thiazole	13.2 (8.8-17.9)	Inactive

al. (1948) to postulate two types of cardiovascular histamine receptors. This hypothesis of two histamine receptors has subsequently been established and it is now known that the depressor response to histamine involves both H_1- and H_2-receptors (Black *et al.*, 1972; Owen & Parsons, 1974; Black *et al.*, 1975).

The experiments reported in this paper show that both types of histamine receptor are involved in the dilatation of peripheral resistance vessels following local injection of histamine. In untreated animals, the histamine dose-response curves were displaced to the right by mepyramine but unaltered by metiamide, indicating that they were due to interaction of histamine with H_1-receptors and were independent of H_2-receptors. The displacement of the dose-response curve by mepyramine achieved a maximum and then further displacement could only be achieved by H_2-receptor blockade with metiamide, indicating that H_2-receptors were also involved in this response. The inability of metiamide to displace the histamine dose-response curve, although it is able to augment maximal displacement by mepyramine, is consistent with the difference in dissociation constant of the H_1- and H_2-receptors, as referred to in the analysis of the depressor responses to histamine (Black *et al.*, 1975).

Involvement of both H_1- and H_2-receptors in the vasodilator responses to histamine has been established in experiments using selective receptor antagonists. Further support for the involvement of both types of receptor has been obtained using selective receptor agonists. Both 2-(2-aminoethyl)pyridine and 2-(2-aminoethyl)thiazole have been shown previously to be selective H_1-receptor agonists (Lee & Jones, 1949; Grossman *et al.*, 1952; Durant *et al.*, 1974). Both compounds caused dose-dependent depressor responses by interaction with H_1-receptors (Owen, 1975) and have now been shown to cause dose-dependent vasodilatation by interaction with H_1-receptors.

2-Methylhistamine has relatively higher agonist activity on H_1-receptors than on H_2-receptors (Black *et al.*, 1972, Durant *et al.*, 1974; Owen, 1975). 2-Methylhistamine up to doses of about 1×10^{-8} mol/kg given locally caused dose-dependent vasodilatation which was due solely to interaction with H_1-receptors. Doses exceeding 1×10^{-8} mol/kg were no longer specific since interaction with H_2-receptors also occurred.

4-Methylhistamine has relatively higher agonist activity on H_2-receptors than on H_1-receptors (Black *et al.*, 1972; Durant *et al.*, 1974; Owen, 1975). 4-Methylhistamine given locally in doses up to 1×10^{-8} mol/kg caused dose-dependent vasodilatation by interaction with H_2-receptors, larger doses also interacted with H_1-receptors.

Each of the histamine-like agonists mimicked the histamine response. Three of the agonists did so by interaction with H_1-receptors and the fourth, 4-methylhistamine, did so by interaction with H_2-receptors. The experiments with the selective agonists therefore support the conclusion that interaction with either H_1- or H_2-receptors can result in vasodilatation.

The potency of the selective agonists relative to histamine was assayed on both H_1- and H_2-receptors in the peripheral vasculature. The values for both 2-methylhistamine and 4-methylhistamine are similar to the values reported by Black *et al.* (1972) on non-vascular histamine receptors. The values for 2-(2-aminoethyl)pyridine and 2-(2-aminoethyl)thiazole on H_1-receptors are similar to the values found by Owen (1975) on cat blood pressure.

The final part of this study indicated that the effects of mepyramine and metiamide on responses to histamine and histamine-like agonists were the consequence of blockade of histamine receptors rather than non-selective effects as neither antagonist alone nor in combination reduced the responses to three non-histamine-like vasodilators, acetycholine, bradykinin and isoprenaline.

In conclusion, histamine-induced vasodilatation in two major peripheral vascular beds has been shown to involve both H_1- and H_2-receptors. Histamine-like responses can be obtained using selective H_1- or H_2-receptor agonists.

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(Received March 10, 1975)

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STUDIES UPON THE MECHANISM BY WHICH ACETYLCHOLINE RELEASES SURPLUS ACETYLCHOLINE IN A SYMPATHETIC GANGLION

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1 Acetylcholine (ACh) releases surplus ACh from the superior cervical ganglion of the cat and the experiments described in this paper tested whether this results from exchange of endogenous ACh with exogenous ACh; the experiments also attempted to characterize pharmacologically the mechanism of this action of ACh.

2 The surplus ACh in the ganglion was radioactively labelled by perfusion of the ganglion with [^{3}H]-choline-Krebs solution containing diisopropylphosphofluoridate, and the release of surplus [^{3}H]-ACh by [^{14}C]-ACh injected close arterially to the ganglion measured. The amount of [^{3}H]-ACh released by [^{14}C]-ACh was 33 ± 5 times greater than was the amount of [^{14}C]-ACh accumulated by ganglia. The amount of exogenous ACh accumulated by ganglia that had first formed surplus ACh was not different from exogenous ACh accumulation by ganglia that had not formed surplus ACh. Thus, it is concluded that surplus ACh release by ACh is not the result of ACh exchange.

3 In other experiments, surplus [^{3}H]-ACh was accumulated in ganglia exposed to physostigmine. Nicotine, pilocarpine or ACh released surplus ACh; the effect of both nicotine and ACh was blocked by hexamethonium, atropine blocked the effect of ACh but not that of nicotine. It is concluded that both nicotinic and muscarinic receptors can be involved in the release of surplus ACh by cholinomimetic agonists.

Introduction

Acetylcholine (ACh) and ACh-like agents such as carbachol and tetramethylammonium can release ACh from the superior cervical ganglion of the cat (McKinstry & Koelle, 1967a; Fellman, 1969; Collier & Katz, 1970). The ACh that is released by ACh is not from the ganglion's normal store of transmitter but is surplus ACh (Brown, Jones, Halliwell & Quilliam, 1970; Collier & Katz, 1970). Surplus ACh is the extra ACh that is synthesized and stored by a ganglion that is exposed to an anticholinesterase agent (Birks & MacIntosh, 1961), this ACh is not immediately available for release by nerve impulses (Birks & MacIntosh, 1961; Collier & Katz, 1971)

The mechanism by which ACh releases surplus ACh is not known. It has been suggested that carbachol (McKinstry & Koelle, 1967a) and tetramethylammonium (Fellman, 1969) release ACh from ganglia by a process of cation exchange; a similar proposal was made much earlier (Renshaw, Green & Ziff, 1938) in an attempt to explain the pharmacological actions of certain choline derivatives, and it was also inferred by Collier & Katz (1970) to explain the release of surplus ACh by ACh. The present experiments were designed to test whether exogenous ACh exchanges with endogenous surplus ACh in ganglia. This was determined directly by labelling ganglionic ACh from [^{3}H]-choline and then measuring the amount of surplus [^{3}H]-ACh released by [^{14}C]-ACh and the amount of [^{14}C]-ACh accumulated by the ganglion. If exogenous ACh exchanged with endogenous ACh, a molecule for molecule exchange of the radioisotopes was expected.

Other experiments attempted to characterize the pharmacology of the process by which ACh releases surplus ACh by testing whether the effect of ACh could be mimicked by muscarinic or nicotinic agonists, and whether ACh-blocking agents block the effect.

Methods

The techniques were similar to those described before (Collier & Lang, 1969; Collier & Katz, 1970).

Ganglion perfusion

Cats were anaesthetized with ethyl chloride followed by ether and then by intravenous chloralose (80 mg/kg). In the experiments in which only one ganglion was to be perfused, the right superior cervical ganglion was prepared for perfusion (Feldberg & Gaddum, 1934), and the left ganglion was removed and used as the control for ACh content In other experiments, both ganglia were prepared for perfusion. The medium used for perfusion was Krebs solution of the following composition (mM): NaCl 120, KCl 4.6, $CaCl_2$ 2.4, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 9.9, it was equilibrated throughout the experiment with 5% CO_2 in O_2 so that the pH was 7 4 at 37° C. In all experiments, perfusion during the first 15 min was with Krebs solution containing choline (10^{-5}M) When the ganglion's surplus ACh was to be radioactively labelled, ganglia (preganglionic trunk cut, but not stimulated) were perfused for 60 min with Krebs solution containing [^{3}H]-choline (10^{-5}M) and an anticholinesterase agent which was either diisopropylphosphofluoridate (DFP, 5×10^{-5}M) or physostigmine (2×10^{-5}M); this procedure labels surplus ACh to a greater specific activity than depot ACh (Collier & Katz, 1971). In experiments in which [^{3}H]-ACh release by injected ACh or an ACh-like agent was measured, the drug was dissolved in 0.2 ml of Krebs solution and injected directly into the arterial cannula. Before injection of the drug it was necessary to wash out the tube leading to the arterial cannula because a little [^{3}H]-choline diffused into its tip during the perfusion with [^{3}H]-choline; to make sure this wash was effective, 0.2 ml of Krebs solution was injected before the drug was tested.

Nerve stimulation

The preganglionic nerve was cut low in the neck and stimulated when necessary with supramaximal rectangular pulses (10 Hz, 0.3 ms, 5-8 V). Contractions of the nictitating membrane were recorded to give some indication of the effectiveness of nerve stimulation or of ganglion stimulation by injected drug.

Preparation of ganglion extracts and bioassay

Ganglia were removed, and extracted with 2.0 ml of 10% trichloroacetic acid (TCA). The TCA was removed by shaking with ether, residual ether was removed by aeration, and separate aliquots of the aqueous phase (pH 4-5) were used for bioassay, for liquid scintillation counting and for separating radioactive ACh from other labelled material.

The ACh content of ganglion extracts and of effluent collected from the stimulated ganglia was measured by bioassay on the blood pressure of the eviscerated cat (MacIntosh & Perry, 1950).

Separation of radioactive materials

In experiments in which ganglia were perfused with [^{3}H]-choline, labelled ACh in ganglion extracts was separated from other ^{3}H-labelled material by the sequential ammonium reineckate and gold chloride precipitation method described before (Collier & Katz, 1971). In the experiments in which ganglia were perfused with [^{14}C]-ACh, labelled ACh in ganglion extracts was separated from labelled acetate by precipitation with ammonium reineckate. In this procedure, unlabelled ACh (1.0 mg) and unlabelled sodium acetate (1.0 mg) were mixed with an aliquot of the ganglion extract, and 2.0 ml of a saturated aqueous solution of ammonium reineckate was added. The reineckate precipitate was washed with saturated ammonium reineckate solution, and ACh was recovered from the precipitate by the anion-exchange resin, Dowex AG2-X8 (Collier & Katz, 1971), ammonium reineckate precipitated ACh (> 95%), but not acetate (< 1%). This procedure was also used to separate [^{14}C]-acetate from unhydrolysed [^{14}C]-ACh in the experiments that tested the hydrolysis of ACh by acetylcholinesterase and to separate [^{14}C]-acetate from [^{3}H]-choline in acetylcholinesterase-treated effluents collected from ganglia in the experiments that measured the release of [^{3}H]-ACh by [^{14}C]-ACh.

Acetylcholine hydrolysis by acetylcholinesterase

When the hydrolysis of ACh by acetylcholinesterase was tested, standard amounts of [^{14}C]-ACh were dissolved in 1.0 ml of Krebs solution without an anticholinesterase or containing DFP; acetylcholinesterase was added and the mixture was incubated at 25° C for 30 minutes. The hydrolysis of ACh was stopped by adding 0.1 ml of 1.0 N HCl, and the amount of ACh remaining was measured after separating [^{14}C]-ACh from [^{14}C]-acetate by precipitation with ammonium reineckate (see above). Aliquots of effluent collected from perfused ganglia were treated in the same way and [^{14}C]-acetate was separated from [^{3}H]-choline by reineckate precipitation.

Determination of radioactivity

Radioactivity in solutions was measured by liquid scintillation spectrometry. In experiments in which a single isotope was to be measured, the solvent system was that described before (Collier & Lang, 1969), and in experiments in which ^{3}H- and ^{14}C-radioactivity was to be measured simultaneously, the solvent system was Aquasol

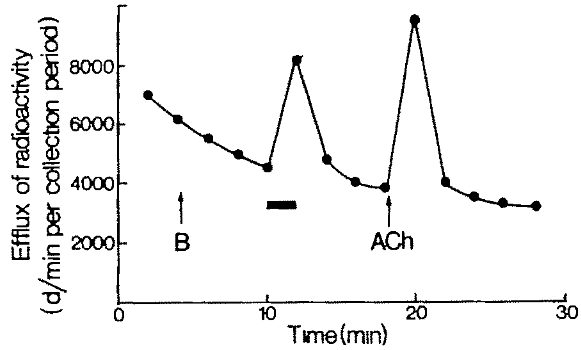

Figure 1 Effect of Krebs solution (0.2 ml indicated by B), of nerve stimulation (10 Hz, during the 2 min period indicated by the horizontal bar), and of injected acetylcholine (ACh, 55 nmol in 0.2 ml Krebs) upon the efflux of radioactivity from the superior cervical ganglion of the cat perfused with diisopropylphosphofluoridate (DFP)-choline-Krebs. The ganglion had previously accumulated labelled surplus ACh during perfusion with $[^3H]$-choline-DFP-Krebs for 60 min; it had then been washed by perfusion with DFP-choline-Krebs for 20 minutes.

(New England Nuclear). Correction for quench and for isotope spill in double-label counting was made, using an internal standard.

Drugs

Compounds used were: methyl-$[^3H]$-choline chloride (50-100 mCi/mmol, New England Nuclear), acetyl-$[1\text{-}^{14}C]$-choline chloride (13.7 mCi/mmol, Amersham/Searle), diisopropylphosphofluoridate (DFP, K&K), acetylcholine chloride (Lematte-Boinot), choline chloride and nicotine hydrogen tartrate (BDH), acetylcholinesterase (*Electrophorus electricus*; 150 units/mg (1 unit hydrolyses 1 μmol ACh/min at 25°C); Schwartz/Mann), physostigmine sulphate, atropine sulphate, and pilocarpine nitrate (Nutritional Biochemicals), and hexamethonium bromide (Poulenc).

Results

The release of surplus acetylcholine by acetylcholine

The previous demonstration (Collier & Katz, 1970) that ACh releases surplus ACh was qualitative, but for the present experiments it was necessary to quantify the release of surplus ACh by ACh. Two experiments in which unlabelled ACh was used to release surplus $[^3H]$-ACh demonstrated how the amount of surplus ACh released could be calculated. In these experiments, the ganglia (not stimulated) were first perfused for 60 min with Krebs solution containing DFP and $[^3H]$-choline to label surplus ACh; perfusion was then switched to DFP-Krebs containing unlabelled choline and when the efflux of radioactivity had declined to a reasonable level (after 20-25 min), the release of $[^3H]$-ACh by preganglionic nerve stimulation (10 Hz for 2 min) and then the release of $[^3H]$-ACh by injected ACh (55 nmol dissolved in 0.2 ml of Krebs solution) was measured. The results of a typical experiment are illustrated in Figure 1; nerve stimulation and injected ACh released radioactivity, but the control injection (0.2 ml of Krebs solution) did not. The other experiment gave similar results. The release of $[^3H]$-ACh by nerve stimulation in these experiments was similar to that measured under similar conditions by Collier & Katz (1971), but was greater than was measured by Collier & Katz (1970) who used slightly different conditions. At

Table 1 Calculation of the amount of surplus acetylcholine (ACh) released by ACh

	Measured		*Calculated*	
	$[^3H]$-ACh (d/min)	*Total ACh (pmol)*		
Released by nerve stimulation	2,800	219	Specific radioactivity of releasable ACh	= 12.8 d min^{-1} pmol^{-1}
Control ganglion		1,648	Total releasable ACh (85%) Total radioactivity of releasable ACh	= 1,401 pmol = 17,934 d/min
Test ganglion	43,900	3,186	Total surplus ACh Radioactivity of surplus ACh	= 1,538 pmol = 25,966 d/min
Released by ACh	5,300		Radioactivity of surplus ACh at test Specific radioactivity of surplus ACh Amount of surplus ACh released	= 31,266 d/min = 20.3 d min^{-1} pmol^{-1} = 261 pmol

the end of the experiment, the perfused ganglion was removed and assayed for total ACh and for [^{3}H]-ACh. The total ACh content of the ganglion effluent collected during nerve stimulation, and the ACh content of the control (not perfused) ganglion were also measured. These measured values were then used to calculate the amount of surplus ACh released by the injected ACh using the procedures summarized in Table 1. The specific radioactivity of the ganglion's releasable ACh was calculated from the measured release of ACh by nerve stimulation; this is valid provided that the period of nerve stimulation is short (Collier, 1969). Multiplication of this value by the total amount of releasable ACh yielded the total radioactivity of releasable ACh; for this calculation, the total ACh content of the test ganglion was taken to be equal to the total ACh content of the control ganglion (Brown & Feldberg, 1936; Feldberg, 1943) and it was assumed that 85% of the total ACh was releasable (Birks & MacIntosh, 1961; Collier & MacIntosh, 1969). Subtraction of the total releasable [^{3}H]-ACh (d/min) from the amount (d/min) of [^{3}H]-ACh measured in the ganglion at the end of the experiment gave the radioactivity of surplus ACh in the ganglion at the end of the experiment, and the sum of this value and the measured release of [^{3}H]-ACh by ACh was taken to be equal to the amount (d/min) of surplus [^{3}H]-ACh in the ganglion at the time that the ACh was injected. The total amount of surplus ACh in the ganglion was calculated from the difference in ACh content of the test and the control ganglia, and this was taken to be the amount of surplus ACh at the time of the ACh injection because the ACh content changes little in the second hour of perfusion with an anticholinesterase (Birks & MacIntosh, 1961; Collier & Katz, 1971). The amount (d/min) of surplus [^{3}H]-ACh in the ganglion divided by the total amount of surplus ACh (pmol) gave the specific activity of surplus ACh, from which the amount (pmol) of ACh released by injected ACh could be calculated. In the two experiments done, the amount of ACh released was 261 pmol and 220 pmol.

The lack of exchange of acetylcholine for surplus acetylcholine

The exchange of exogenous ACh with endogenous surplus [^{3}H]-ACh was tested by experiments like those described above; surplus ACh release was evoked by injection of [^{14}C]-ACh and the amount of [^{14}C]-ACh accumulated by the ganglion at the end of the experiment was also measured. Preliminary experiments of this type showed that the [^{3}H]-ACh content of ganglion extracts could readily be measured in the presence of the accumulated [^{14}C]-ACh by standard double-isotope methods. However, the presence of an excess of ^{14}C- to ^{3}H-radioactivity in the effluent collected from the ganglion immediately following the injection of [^{14}C]-ACh precluded the use of standard double-isotope counting techniques for measurement of the released [^{3}H]-ACh. This problem was overcome by using acetyl-[1-^{14}C]-choline and hydrolysing the ACh in the effluent collected from the ganglion with acetylcholinesterase. The released [^{3}H]-ACh, which had been synthesized *in situ* from precursor choline, was N-methyl-labelled whereas the exogenous ACh was acetyl-labelled, hydrolysis yielded [^{3}H]-choline from released ACh and [^{14}C]-acetate from injected ACh and these two labelled materials were easily separated by precipitation with ammonium reineckate (see methods section). Because the irreversible anticholinesterase agent, DFP, was used in these experiments it could safely be omitted from the perfusion fluid during the 2 min period that immediately followed the injection of [^{14}C]-ACh; the concentration of DFP in fluid collected would then be $< 5 \times 10^{-5}$ M. The maximum concentration of ACh in the collected effluent was calculated to be 6×10^{-5} M. The results of *in vitro* experiments that measured the hydrolysis of ACh by acetylcholinesterase in the presence or in the absence of DFP showed that 5.4 units/ml of the enzyme hydrolysed all of the added ACh even in the presence of DFP; this concentration of acetylcholinesterase was used in the experiments that tested the release of ACh by [^{14}C]-ACh.

The procedures described above were then combined to test the exchange of exogenous [^{14}C]-ACh for endogenous surplus [^{3}H]-ACh. Injected [^{14}C]-ACh released [^{3}H]-radioactivity and the calculations described above (Table 1) were made for these experiments to provide an estimate of the amount of surplus [^{3}H]-ACh released. The amount of [^{14}C]-ACh accumulated was calculated from the ^{14}C-radioactivity measured in the ganglion extract and the specific activity of ACh used. Table 2 summarizes the results of three such experiments; the amount of ACh released by ACh was 24-40 times greater than the amount of ACh accumulated. Thus, exogenous ACh does not release endogenous ACh by a mechanism of ACh exchange.

This conclusion that exogenous ACh does not exchange with endogenous surplus ACh was supported by other experiments that measured ACh accumulation by ganglia that had, or had not, formed surplus ACh. In these experiments, both superior cervical ganglia were perfused. One ganglion was perfused for 60 min with Krebs

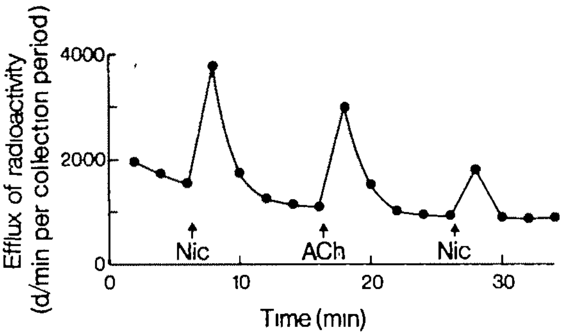

Figure 2 Effect of 50 nmol of nicotine (NIC) and of acetylcholine (ACh) on the efflux of radioactivity from the superior cervical ganglion of the cat perfused with Krebs solution containing physostigmine and choline. The ganglion had previously accumulated surplus [^{3}H]-ACh during perfusion with [^{3}H]-choline-Krebs containing physostigmine for 60 min, and had then been washed by perfusion with physostigmine-choline-Krebs for 30 minutes

solution containing DFP and choline (surplus ACh accumulated) and the other ganglion was perfused with choline-Krebs solution (no anticholinesterase, therefore, no surplus ACh accumulated). Both ganglia were then perfused for 90 min with choline-free Krebs solution containing DFP and perfusion was switched to the same medium containing [^{14}C]-ACh (2.8×10^{-6} M) for 5 min every 10 minutes. At the end of the experiment, the ganglia were removed and their [^{14}C]-ACh content and their total ACh content was measured The results of these experiments are summarized in Table 3. The ganglia that had been exposed to DFP and choline during the first 60 min of perfusion contained 45% more ACh, but no more [^{14}C]-ACh than did the ganglia that had not been exposed to DFP in the first 60 min of perfusion.

The release of surplus acetylcholine by acetylcholine-like drugs

To test whether the release of surplus ACh by ACh involved an action of the drug upon nicotinic or muscarinic receptors, the release of surplus ACh by nicotine or by pilocarpine was tested in five experiments. In these experiments, surplus [^{3}H]-ACh was allowed to accumulate in ganglia perfused with [^{3}H]-choline in the presence of physostigmine, perfusion was then continued with Krebs solution containing unlabelled choline and physostigmine and the effect of the drugs upon the release of [^{3}H]-ACh was measured by injecting them (50 nmol) into the arterial cannula. Both nicotine and pilocarpine, like ACh, released radioactivity (Figures 2 and 3), and the extra ACh

Table 2 Comparison of the amount of surplus [^{3}H]-acetylcholine (ACh) released by [^{14}C]-ACh to the amount of [^{14}C]-ACh accumulated by ganglia

	[^{3}H]-ACh released		*[^{14}C]-ACh accumulated*		*[^{3}H]-ACh released/ [^{14}C]-ACh accumulated*
Expt.	*d/min*	*pmol*	*d/min*	*pmol*	
1	4132	203	170	5.7	36
2	4800	308	380	12.7	24
3	5600	292	220	7.3	40
Mean ± s.e.	4844 ± 424	268 ± 32	257 ± 63	8.6 ± 2.1	33 ± 5

Table 3 [^{14}C]acetylcholine (ACh) uptake by ganglia that had (test) or had not (control) previously accumulated surplus ACh

	[^{14}C]-ACh (d/min)		*Total ACh content (pmol)*	
Experiment	*Test ganglion*	*Control ganglion*	*Test ganglion*	*Control ganglion*
1	478	511	2198	1538
2	909	969	2747	1648
3	608	596	1813	1483
Mean ± s.e.	665 ± 128	692 ± 141	2253 ± 262	1556 ± 53

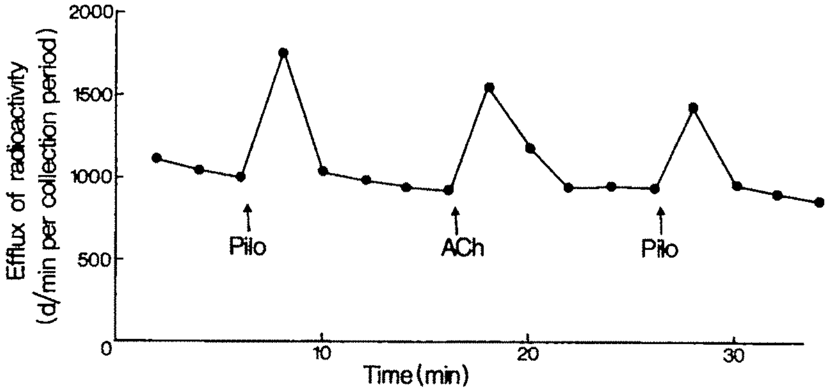

Figure 3 Effect 50 nmol of pilocarpine (Pilo) and of acetylcholine (ACh) on the efflux of radioactivity from the superior cervical ganglion of the cat. Experimental conditions were the same as for Figure 2.

released by the drugs was [^{3}H]-ACh (see methods section). The nictitating membrane contracted in response to ACh or to nicotine, but little if any contracture resulted after injecting pilocarpine.

The effect of acetylcholine-blocking agents upon the release of surplus acetylcholine induced by acetylcholine or by nicotine

In these experiments, surplus [^{3}H]-ACh was formed by ganglia in the presence of physostigmine, perfusion was continued with medium containing physostigmine and non-radioactive choline and the release of surplus [^{3}H]-ACh by the agonist was tested before, during, and after exposing the ganglion to the antagonist. Four experiments showed that atropine (5×10^{-5} M) completely blocked the release of ACh induced by ACh (Figure 4a); the injection of ACh in the presence of atropine still induced a contraction of the nictitating membrane. Two experiments showed that atropine (2×10^{-4} M) did not block the release of ACh induced by nicotine (Figure 4b). Two experiments showed that hexamethanium (2×10^{-4} M) clearly reduced the release of ACh induced by nicotine (Figure 5a). Five experiments tested whether hexamethonium (2×10^{-4} M) blocked the release of surplus ACh induced by ACh; in four of these release was almost completely abolished (Figure 5b), but in the other, release was not clearly blocked. In all experiments, hexamethonium almost completely blocked the contraction of the nictitating membrane that resulted from injecting nicotine or ACh.

Discussion

The present experiments were designed to test whether exogenous ACh releases endogenous ACh from the cat superior cervical ganglion by a process of ACh exchange. The most direct test of the mechanism was to compare the amount of exogenous [^{14}C]-ACh accumulated by ganglia with the amount of endogenous [^{3}H]-ACh released from ganglia by the [^{14}C]-ACh. If endogenous ACh is released as the result of ACh exchange, a molecule for molecule exchange of the isotopes would be expected in these experiments. The ratio of [^{3}H]-ACh released to [^{14}C]-ACh accumulated was calculated to be 33. The calculation of this ratio of ACh released to ACh accumulated assumed that exogenous ACh releases surplus ACh and not transmitter that is available for release by nerve impulses; this assumption is probably correct (Brown *et al.*, 1970; Collier & Katz, 1970). If the calculation of the amount of [^{3}H]-ACh released by ACh had been made from the specific radioactivity of total tissue ACh, or from the specific activity of releasable ACh, the ratio of ACh released to ACh accumulated would have been greater than the ratio calculated, because the conditions of the experiments were such that surplus ACh was labelled to higher specific radioactivity than was releasable ACh.

The ratio of the amount of surplus [^{3}H]-ACh released from nerve endings to the amount of exogenous [^{14}C]-ACh accumulated by nerve endings would also be greater than the ratio that was calculated if part of the [^{14}C]-ACh accumulation measured was the result of

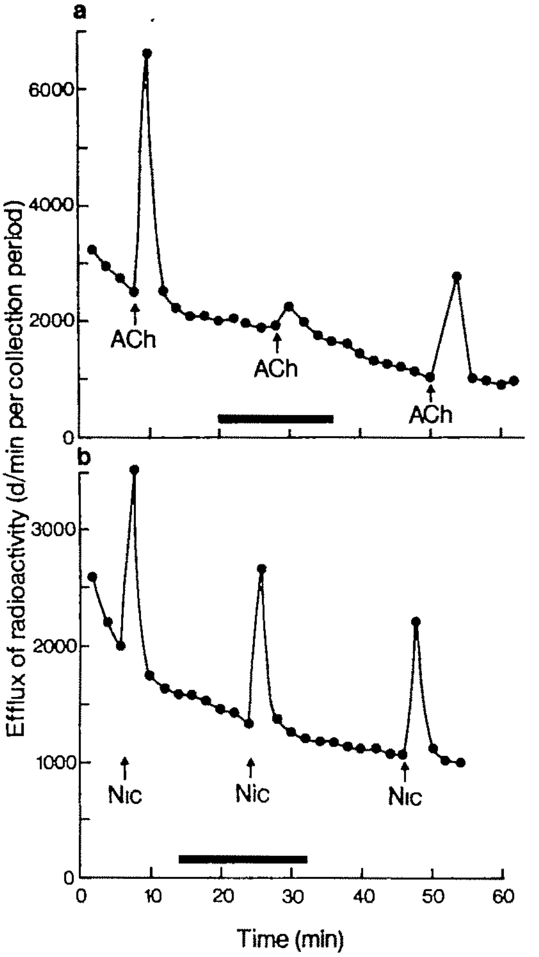

Figure 4 Effects of atropine (a) 50 μM and (b) 200 μM (present during the time indicated by the horizontal bars) on the release of surplus [^{3}H]-acetylcholine induced by 50 nmol of (a) acetylcholine (ACh) and (b) nicotine (Nic). Experimental conditions were the same as for Figure 2; (a) and (b) are separate experiments.

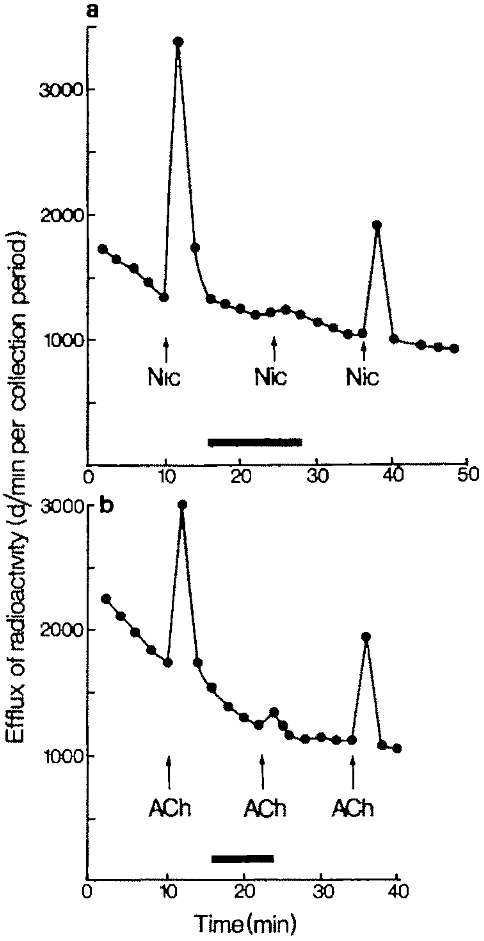

Figure 5 Effects of hexamethonium 200 μM (present during the time indicated by the horizontal bars) on the release of surplus [^{3}H]-acetylcholine induced by 50 nmol of (a) nicotine (Nic) and (b) acetylcholine (ACh). Experimental conditions were the same as for Figure 2; (a) and (b) are separate experiments.

non-specific binding of ACh or represented [^{14}C]-ACh uptake into structures other than preganglionic nerve endings. Other experiments (Katz, Salehmoghaddam & Collier, 1973) have shown that chronic decentralization does not reduce the amount of exogenous ACh accumulated by ganglia exposed to ACh for 90 min, which indicates that under those conditions ACh accumulation was primarily by structures other than cholinergic nerve terminals (see also Kuhar & Simon, 1974). In the present experiments, the exogenous ACh was injected close to the ganglion so that the time of exposure of the tissue to [^{14}C]-ACh was as short as possible; under these conditions, the site at which exogenous ACh accumulates is not known.

There is now evidence suggesting that mammalian brain and the electric organ of *Torpedo* can synthesize from radioactive choline a small pool of ACh that is of much greater specific radioactivity than is the rest of the tissue's ACh (Barker, Dowdall, Essman & Whittaker, 1970; Richter & Marchbanks, 1971; Marchbanks & Israël, 1971, 1972; Barker, Dowdall & Whittaker, 1972). It could be argued that such a pool of endogenous ACh might exchange with exogenous ACh, but if this were so in the present experiments, the [^{3}H]-ACh released (4844 d/min) in exchange for the 8.6 pmol of [^{14}C]-ACh accumulated (see Table 2) would have to have been from a pool of ACh with a specific radioactivity of 563 d min^{-1} pmol^{-1}. It is not possible that ACh of this specific radioactivity could have been synthesized in these experiments because the specific radioactivity of choline used was 222 d min^{-1} pmol^{-1}.

The results of experiments which measured the uptake of exogenous ACh by ganglia that had, or had not, first accumulated surplus ACh were consistent with the conclusion that surplus ACh release by ACh does not result from ACh exchange. If the exchange occurred, it might be expected that accumulation of exogenous ACh would have been greater in ganglia with surplus ACh. This was not so.

The previous suggestion (Collier & Katz, 1970) that the mechanism by which ACh releases ACh from ganglia might be similar to that by which certain amines release noradrenaline from sympathetic nerves appears to be wrong, for example, under appropriate conditions, a molecule for molecule exchange of metaraminol for noradrenaline can be demonstrated (Porter, Torchiana, Totaro & Stone, 1967).

The results of McKinstry & Koelle's (1967b) experiments suggest that ACh-blocking agents block the release of surplus ACh release evoked by carbachol, as if release might involve presynaptic cholinoceptors. The present experiments are consistent with this, but the mechanism involved could not be clearly defined as nicotinic or muscarinic. Both pilocarpine, which exerts primarily muscarinic effects, and nicotine, which exerts nicotinic effects, released surplus ACh; thus activation of either type of receptor appears to evoke ACh release. The effect of ACh in releasing surplus ACh might, therefore, be expected to be reduced, but not completely blocked by either atropine, which is anti-muscarinic, or by hexamethonium, which is anti-nicotinic; however, either antagonist alone effectively blocked the effect of ACh. If the presynaptic cholinoceptor involved in the phenomenon of surplus ACh release by ACh were sensitive to blockade by either atropine or hexamethonium, and if nicotine mimics the action of ACh, either antagonist might be expected to block this action of nicotine; however, only hexamethonium prevented the effect of nicotine. Further speculation about the mechanism by which ACh releases surplus ACh is unwarranted without more experimental evidence.

This work was supported by the MRC (Canada) and the DRB (Canada). We are grateful to L. Bonet and J. Elliott for technical help and to D. Ilson for reading the manuscript.

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(Received April 21, 1975.
Revised June 3, 1975.)

Br. J. Pharmac. (1975), 55, 199-212

MEMBRANE POTENTIAL CHANGES INDUCED BY 5-HYDROXYTRYPTAMINE IN THE RABBIT SUPERIOR CERVICAL GANGLION

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1 Changes in resting membrane potential induced by 5-hydroxytryptamine (5-HT) have been measured in the excised ganglion by the sucrose-gap technique.

2 5-HT produced a rapid depolarization, the threshold concentration for depolarization being around 10 μM. With concentrations of 100 μM or greater, repolarization began during the course of the superfusion; this was followed by prolonged tachyphylaxis.

3 Tachyphylaxis was largely avoided by making injections into the superfusion stream. Standard injections of 0.2 μmol 5-HT dissolved in 0.2 ml of Krebs solution were used routinely and could be given at 20-30 min intervals to evoke relatively constant responses.

4 The response to an injection consisted of a rapid depolarization, followed by a rapid repolarization and subsequent after-hyperpolarization. The threshold quantity for depolarization was around 0.01 μmol, while the ED_{50} estimated from 6 dose-response curves was 0.12 ± 0.02 μmol (mean ± s.e. mean).

5 Injections of 5-HT (0.2 μmol), choline (10 μmol) and acetylcholine (9.9 μmol) produced depolarizations of similar magnitude.

6 Monoamine oxidase inhibitors failed to alter substantially the amplitude of depolarizations to 5-HT.

7 5-HT depolarizations were unaltered in amplitude when the impermeant anion benzenesulphonate was substituted for the chloride ion in Krebs solution, but were initially markedly reduced in amplitude in a sodium-deficient medium; some recovery of the response subsequently occurred. The depolarization which persisted in sodium-deficient solutions was much reduced or abolished when calcium ions were then removed from the superfusion medium. Removal of either calcium ions alone or potassium ions from the superfusion fluid did not reduce depolarization amplitude.

8 The after-hyperpolarization was abolished in sodium-deficient solutions, usually increased in potassium-free solutions, reduced or abolished by ouabain or nicotine, but unaffected by calcium-free solutions.

9 A depolarizing action of 5-HT on presynaptic terminals in the ganglion appears probable.

Introduction

The excitatory action of 5-hydroxytryptamine (5-HT) on the guinea-pig ileum described by Gaddum (1953) was subsequently ascribed to activation of 5-HT receptors located on neurones (Gaddum & Picarelli, 1957). The receptors were designated M receptors and it was noted that tachyphylaxis was readily induced. These receptors could be differentiated from the 5-HT receptors of smooth muscle in terms of the antagonists which affected them. More recently there have been a number of reports of the excitatory action of 5-HT on ganglion cells of the autonomic nervous system (see Haefely, 1972, 1974); occasionally, inhibitory actions have also been observed (see Wallis & Woodward, 1974). de Groat & Volle (1966), Jaramillo & Volle (1968), Machova & Boska (1969) and Haefely (1974) described changes in ganglionic surface potential induced by injection of the amine into the blood supply of the cat superior cervical ganglion, while Wallis & Woodward (1973, 1974) reported that 5-HT evoked a rapid depolarization in the excised superior cervical ganglion of the rabbit. Recent studies on the cat superior cervical ganglion have suggested that 5-HT may mediate three distinct responses involving different 5-HT receptors a depressant action and two types of excitation (de Groat & Lalley, 1973). Haefely (1974) also described separate depressant and excitatory actions in the same ganglion. He suggested that 5-HT receptors may be located both presynaptically and postsynaptically.

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Despite these studies the nature of ganglion 5-HT receptors and the ionic basis of the membrane potential changes are still far from clear, while quantitative analysis of the potential changes induced by 5-HT has not usually been attempted.

This paper describes experiments in which the effects of 5-HT on membrane potential were examined in the excised superior cervical ganglion by the sucrose-gap technique. The aim of the experiments was to characterize more fully some of the properties of peripheral neuronal 5-HT receptors. A preliminary account of this work has been given to the British Pharmacological Society (Wallis & Woodward, 1973).

Methods

Preparation

Rabbits were anaesthetized with urethane (1.5-2 g/kg, i.p. as a 50% w/v solution). The superior cervical ganglion was removed, together with several cm of the cervical sympathetic nerve and as great a length as possible of the internal carotid nerve. The ganglia were prepared for insertion into the sucrose-gap apparatus, in which the sucrose compartment was separated by membranes, by the method previously described (Kosterlitz & Wallis, 1966; Kosterlitz, Lees & Wallis, 1968; Wallis, Lees & Kosterlitz, 1975). With this method, stable recordings may be made over periods of up to 5 hours. Potential changes were displayed on a potentiometric chart recorder (Servoscribe R.E. 511.20). The amplitude of the potential change was measured from a projection of the baseline preceding the response. Baseline drift was variable but tended to be greater in those experiments in which changes were made in the ionic composition of the superfusion medium. Flow rate over the ganglion was 2-3 ml/minute.

Solutions

All solutions were made up from distilled water passed through a deionizer. The Krebs solution used to superfuse the ganglion had the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4$ 1.2 and glucose 11; it was gassed with 5% CO_2 and 95% O_2. In experiments in which the effects of changes in ionic composition of the superfusion medium on 5-HT depolarizations were examined, the following solutions were used: (1) low-chloride Krebs in which NaCl was replaced with an equivalent amount of sodium benzenesulphonate ($C_6H_5 . SO_3Na$); (2) low-sodium/low-chloride Krebs in which all NaCl was replaced with an equivalent amount of sucrose; (3) sodium-free solution in which the NaCl and $NaHCO_3$ were omitted from Krebs solution and replaced with an equivalent amount of Tris HCl, pH being adjusted to 7.4 by the addition of HCl; (4) calcium-free Krebs in which the $CaCl_2$ was omitted from normal Krebs and the small change in osmolarity ignored; (5) calcium-rich Krebs in which the $CaCl_2$ concentration was raised from 2.54 to 5.08 mM and the small change in osmolarity ignored; (6) potassium-free Krebs in which KCl was replaced with an equivalent amount of NaCl and (7) potassium-rich Krebs in which KCl replaced equivalent amounts of NaCl (see Wallis *et al.*, 1975). The concentration of the sucrose solution superfusing part of the internal carotid nerve was 315 mM and taken to be isotonic. Experiments were carried out at temperatures between 20 and 22° C.

Drugs

The drugs used were 5-hydroxytryptamine creatinine sulphate (5-HT), acetylcholine chloride (ACh), choline chloride, physostigmine (eserine) sulphate, hexamethonium bromide, nicotine hydrogen tartrate, iproniazid phosphate, harmine hydrochloride, strophanthin G (ouabain) and dinitrophenol. The concentrations (μM) and quantities of drugs injected (μmol) refer to the salts.

Results

Depolarization by continuous superfusion or by injections of 5-hydroxytryptamine into the superfusion stream

In confirmation of a number of previous reports (see introductory section), we have found that 5-HT is a potent depolarizing agent of sympathetic ganglia. When a solution of 5-HT (100 μM) superfused the ganglion, a rapid depolarization occurred (Figure 1c). In the experiment illustrated, a concentration of 10 μM also induced a depolarization (Figure 1b), while a concentration of 1 μM appeared to be below threshold for a membrane potential change (Figure 1a). In most experiments the threshold concentration for

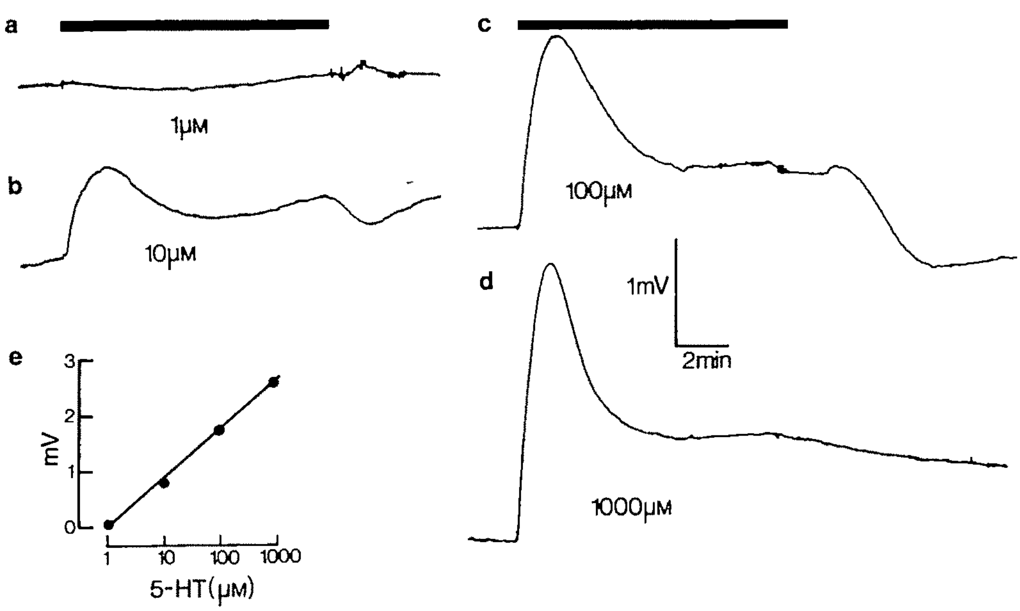

Figure 1 Effect on resting potential of rabbit superior cervical ganglion of superfusion with various concentrations of 5-hydroxytryptamine (5-HT). (a-d) Chart records, depolarization upwards. Solid bars, superfusion with Krebs solution containing 5-HT: (a) 1 μM, (b) 10 μM, (c) 100 μM, (d) 1000 μM. Initial exposure was to 10 μM; 1 μM was tested after washing for 75 min, then 100 μM after washing for 35 min and finally 1000 μM after washing for 45 minutes. In (e) amplitude of peak depolarization (mV) is plotted on a semilogarithmic scale against concentration (μM) of 5-HT.

depolarization was around 10 μM, while a concentration of 100 μM consistently produced depolarization. With the latter concentration and with higher concentrations, it was noticeable that repolarization began during the course of superfusion with the drug. On returning to superfusion with normal Krebs solution, there were often indications that an after-hyperpolarization ensued (e.g. Figure 1c). As reported previously (Wallis & Woodward, 1974), the transmitted ganglionic action potential was depressed during depolarization by 5-HT. Although the amplitude of the peak depolarization evoked by 5-HT was concentration-dependent (Figure 1e), in most experiments the tachyphylaxis which followed an initial exposure made subsequent determinations of the depolarizing action difficult, unless long periods of washing with Krebs solution intervened. However, tachyphylaxis was largely avoided if injections of 5-HT were made into the superfusion stream. This method makes estimation of the concentration of 5-HT at the ganglion hazardous; injection of 0.2 ml of dye at the end of the experiment showed that the material was dispersed in a volume of about 5 ml. Preliminary experiments suggested that in any particular preparation an injection of 0.2 μmol (81 μg) 5-HT dissolved in a volume of 0.2 ml Krebs solution produced a depolarization which was 50% maximal or larger. Injections of 5-HT were given at 20 or 30 min intervals and elicited depolarizations which were relatively constant (Figure 2), although some decline occurred over a period of hours. The response consisted of a depolarization of rapid onset and an almost equally rapid repolarization, followed by an after-hyperpolarization. Injections of 0.2 μmol 5-HT produced relatively consistent potential changes, as can be seen from the standard errors in Table 1, first column. Further, successive responses from the same preparation were well maintained, even though there was a slight decline in depolarization amplitude and in the other parameters of the response (Table 1). Comparing the first with the third response, these differences are statistically significant.

Changes in the volume of the Krebs solution in which a given amount of 5-HT was dissolved had little effect on the magnitude of the depolarization, except that small volumes (i.e. less than 0.1 ml) were proportionately less effective. To construct dose-response curves, the smaller quantities of 5-HT were injected at greater dilution. In two such experiments it was possible to obtain curves based on at least eight responses

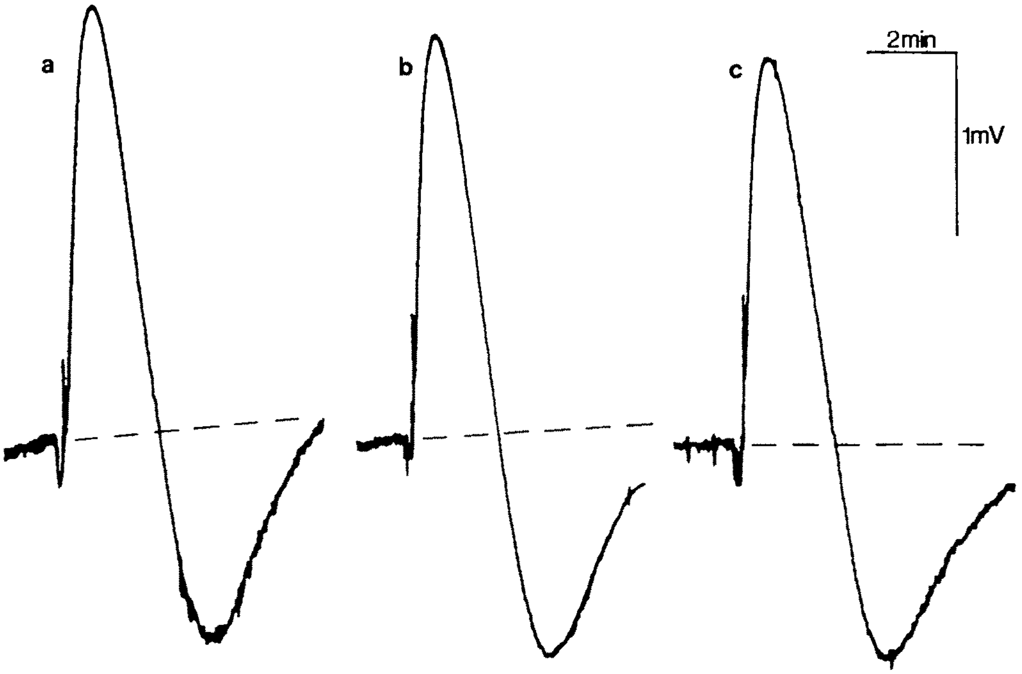

Figure 2 Resting potential changes of rabbit superior cervical ganglion in response to injections of 5-hydroxytryptamine (5-HT) into the superfusion stream. 5-HT 0.2 μmol (81 μg) dissolved in 0.2 ml Krebs solution injected at 30 min intervals. Chart records, depolarization upwards; note initial downward deflection of pen is injection artefact. (a) second, (b) third and (c) fourth response to 5-HT. The dashed lines in this and subsequent figures indicate baseline drift estimated over several min prior to the response.

(Figure 3). The amplitude and the area of the depolarization have been plotted against the quantity of 5-HT injected. An approximately linear relationship exists between depolarization amplitude and the logarithm of the quantity of 5-HT over a range of 5-HT values; for larger quantities of 5-HT the amplitude reached a plateau (Figure 3a and b). The area of the depolarization, however, tended to increase without clearly reaching a plateau because, for larger quantities of 5-HT, the duration of the response also increased. The threshold quantity for the depolarizing action of 5-HT appeared to be around 0.01 μmol (5 μg). In some preparations, it proved difficult because of the time involved to obtain sufficient responses for a full curve and, in general, it was not possible

Table 1. Characteristics of 1st, 2nd and 3rd responses to injections of 0.2 μmol 5-hydroxytryptamine

Injection	*Depolarization (mV)*	*Rate of depolarization (mV/min)*	*Duration of depolarization (min)*	*Rate of repolarization (mV/min)*	*Hyperpolarization (mV)*
1st $n = 28$	1.91 ± 0.10	3.20 ± 0.28	2.73 ± 0.10	1.66 ± 0.14	0.97 ± 0.10
2nd $n = 30$	1.83 ± 0.12	2.86 ± 0.23	3.13 ± 0.14	1.29 ± 0.09	0.87 ± 0.09
3rd $n = 25$	1.59 ± 0.12 $P < 0.01$	2.72 ± 0.27 $P < 0.05$	3.07 ± 0.14 $P < 0.01$	1.13 ± 0.10 $P < 0.001$	0.77 ± 0.11 $P < 0.001$

Values show mean ± s.e. mean; where baseline instability occurred the response was not analysed, hence the variability in n. Values of P refer to comparison of 1st and 3rd responses. Successive injections at 20-30 min intervals.

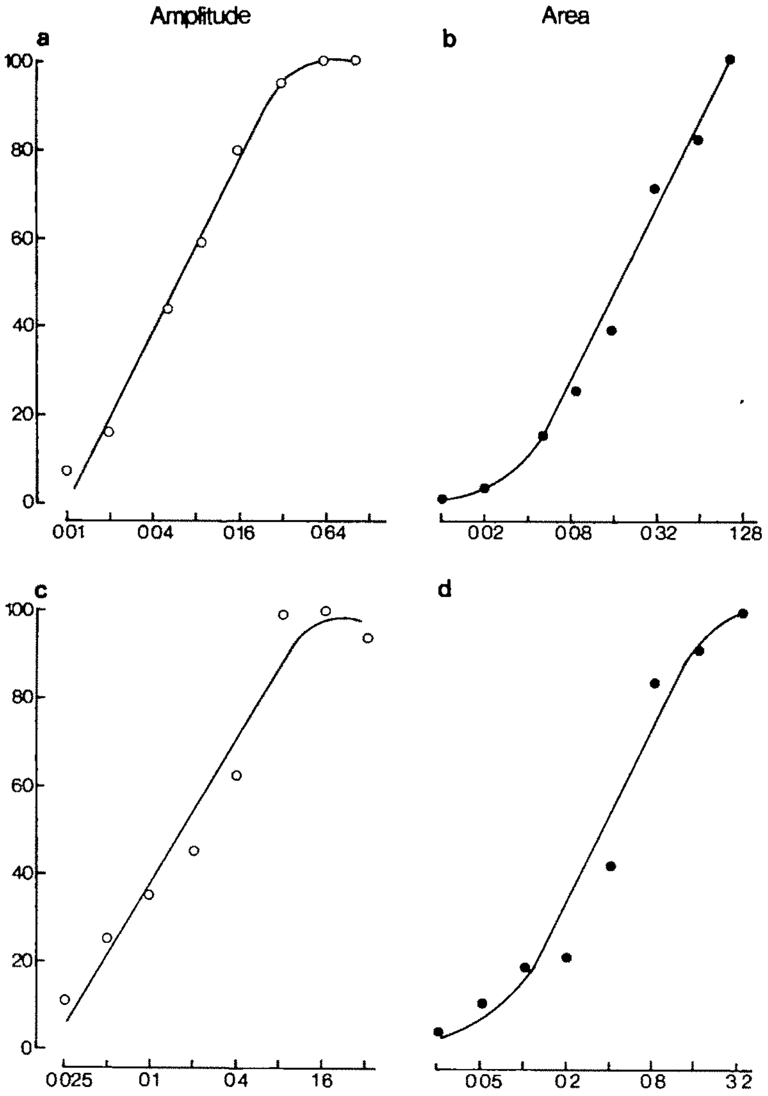

Figure 3 Relationship between quantity of 5-hydroxytryptamine (5-HT) and amplitude and area of the depolarization in two experiments. Abscissae: μmol 5-HT injected into the superfusion stream in a volume ranging from 0.1-0.65 ml, logarithmic scale; ordinates: depolarization amplitude or depolarization area expressed as a percentage of the maximal response. (a) and (b) are curves from one preparation, (c) and (d) curves from another.

to repeat observations for a second curve on the same preparation. However, in 6 preparations the ED_{50} values for the depolarizing action of 5-HT could be estimated and ranged from 0.06-0.22 μmol (24-88 μg) with a mean of 0.12 (49 μg) ± 0.02 μmol (mean ± s.e. mean).

The after-hyperpolarizations which follow depolarization were also concentration-dependent, at least over a certain range, but the tendency for the after-hyperpolarizations to decline with time made it difficult to construct full dose-response curves.

Comparison with the depolarizing action of acetylcholine and choline

The depolarizing action of 5-HT on ganglion cells appeared to be comparable to that of acetyl-

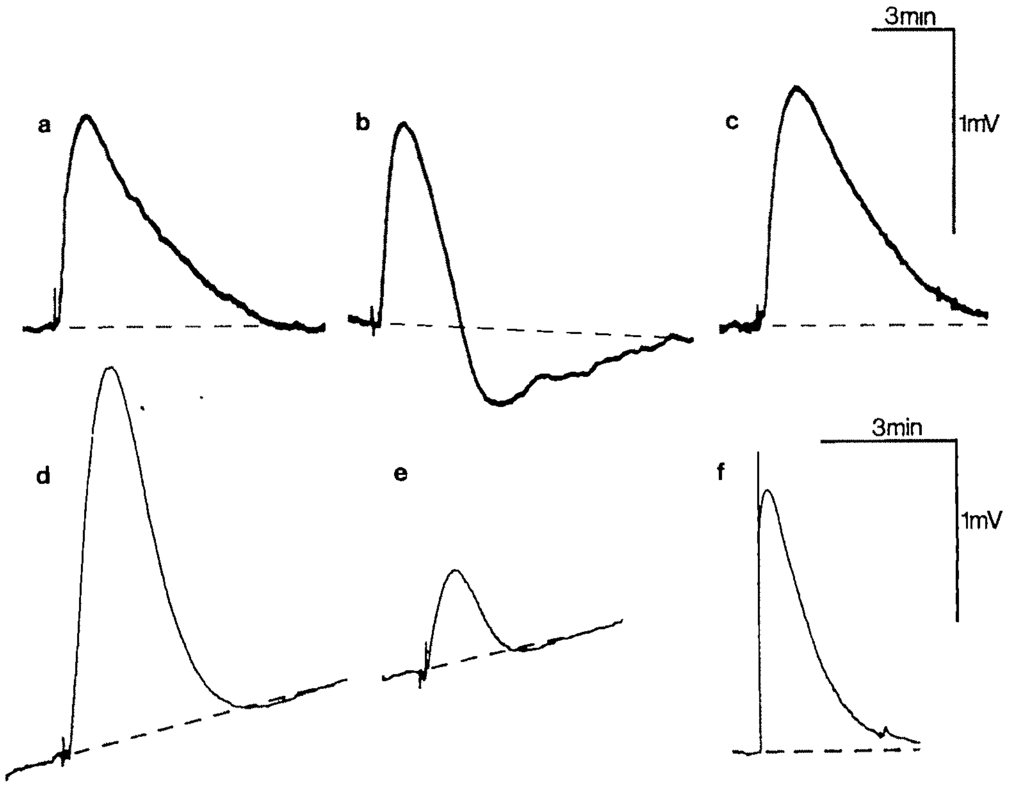

Figure 4 Depolarizations by 5-hydroxytryptamine (5-HT), acetylcholine (ACh) and choline and the effect of an anticholinesterase. (a) injection of 9.9 μmol ACh, (b) injection of 0.2 μmol 5-HT, (c) injection of 10 μmol choline, (a-c) from same preparation, (d-f) in the presence of 30 μM physostigmine in a second preparation, (d) injection of 0 2 μmol ACh, (e) injection of 0.2 μmol 5-HT, (f) LN wave following a 1 s train of stimuli at a frequency of 10 Hz to the preganglionic nerve. Spike amplitude is attenuated by the recording system.

choline (ACh) or choline (Figure 4), although the amplitude of a maximal depolarization produced by 5-HT was generally smaller than a maximal depolarization induced by ACh. ACh applied by superfusion is largely hydrolysed to choline before it can depolarize the ganglion cells (Kosterlitz *et al.*, 1968). In a preparation untreated with anti-cholinesterase, injections into the superfusion stream of ACh (9.9 μmol, 1800 μg), 5-HT (0.2 μmol, 81 μg) and choline (10 μmol, 1400 μg) produced depolarizations of similar magnitude displaying similar rates of depolarization (Figure 4a-c). After-hyperpolarizations were only consistently observed, however, in response to 5-HT. The large quantity of ACh required presumably reflects the high rate of hydrolysis of this substance, for in preparations treated with physostigmine (30 μM), much smaller amounts of ACh (e.g. 0.2 μmol) produced large depolarizations of rapid onset (Figure 4d), while the late depolarization (LN wave) which results from the muscarinic action of ACh released by orthodromic stimulation was also greatly potentiated (Figure 4f). In contrast, the sensitivity to 5-HT appeared to be depressed in the presence of physostigmine for 5-HT evoked only small depolarizations (Figure 4e).

It was of interest to know whether 5-HT might be destroyed enzymatically before the amine could act on the ganglion cells. In studies on the isolated rat stomach strip, Vane (1959) found that monoamine oxidase activity altered the relative potency of tryptamine and 5-HT, probably because of different rates of access to the enzyme. However, in the rabbit superior cervical ganglion, neither the amplitude of the depolarization induced by 5-HT nor the quantity required to produce a depolarization were much affected by pretreatment of the ganglion with iproniazid (2 mM), although the duration of the depolariza-

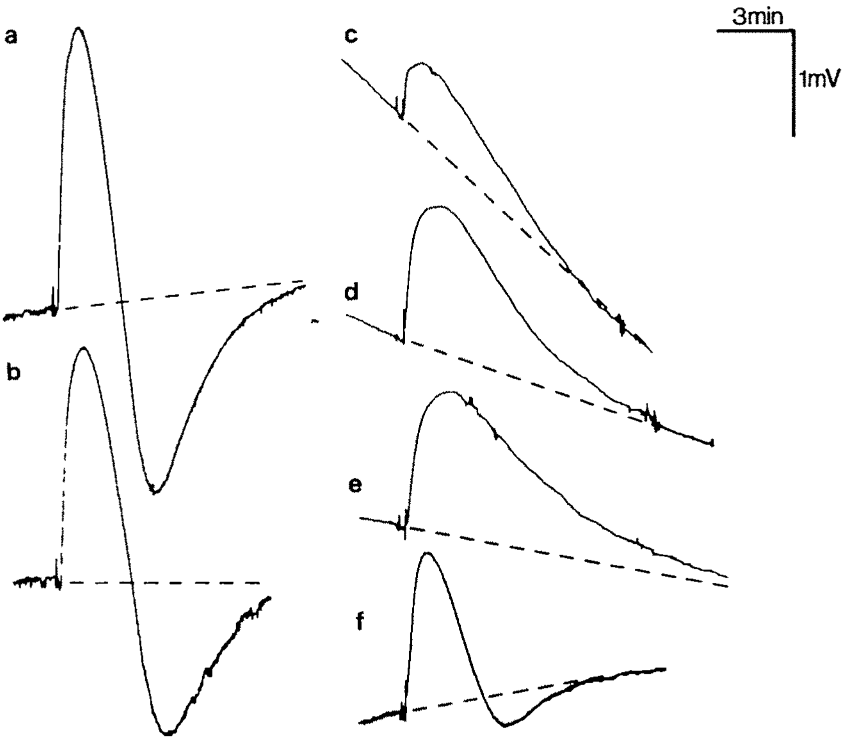

Figure 5 Effect of changes in sodium ion concentration of superfusion medium on 5-hydroxytryptamine (5-HT) depolarizations. (a-f) responses to injection of 0.2 μmol 5-HT. (a) and (b) controls, (c) 19 min, (d) 43 min and (e) 92 min after superfusing with sodium-free Krebs solution, (f) 41 min after returning to normal Krebs solution.

tions was increased. This concentration of iproniazid is 1.5 times that used by Gertner, Paasonen & Giarman (1959) in their studies on cat ganglia. Harmine (40 μM) also failed to alter the amplitude of the response to the standard injection of 5-HT (0.2 μmol).

Ionic basis of the depolarization

Both γ-aminobutyric acid (GABA) and 5-HT have actions on cat ganglia and picrotoxin, a GABA antagonist, blocks the excitatory effects of 5-HT (de Groat, 1970). It was therefore possible that the ganglionic actions of 5-HT and GABA might be due to activation of the same receptors. The potential change induced in the rat ganglion cell membrane by GABA has been studied in detail (Bowery & Brown, 1972, 1974; Adams & Brown, 1973) and is due to an increased chloride ion permeability. In our experiments, 5-HT depolarizations were of similar amplitude when the sodium chloride of the superfusion medium was entirely replaced with sodium benzenesulphonate, the anion of which is impermeant. Unlike the situation in rat ganglia, where successive GABA depolarizations in the absence of external chloride become progressively smaller as the intracellular concentration of chloride ions falls (Adams & Brown, 1973), successive 5-HT depolarizations showed no signs of diminution in low-chloride Krebs in our experiments.

The similarity of the depolarizations to ACh and 5-HT (Figure 4) suggested that the ionic basis of the two phenomena might be similar, i.e. an increased permeability to cations (cf. Koketsu, 1969). In a sodium-free superfusion medium (Figure 5), depolarizations evoked by 5-HT were initially very much reduced in amplitude. The rate of depolarization was slightly reduced and the rate of repolarization considerably reduced; the after-hyperpolarization was usually abolished. On removing the sodium ions from the superfusion medium, a rapid hyperpolarization was recorded followed by a prolonged further hyperpolarization lasting many minutes; 19 min after changing to the sodium-free medium (Figure 5c), the 5-HT depolarization was superimposed on this slow hyperpolarization. Potential changes recorded on altering the ionic composition of the medium are partly due to changes in junction potentials (Wallis *et al.*, 1975) which could account for the rapid hyperpolarization observed; the slow hyperpolarization may be largely a membrane potential change induced by the altered ionic environment of the ganglion cells. In each of 8 experiments, reducing $[Na]_o$ led first to a reduction of the depolarization and the reduction was most pronounced in sodium-free solution; this was followed by a substantial recovery while still superfusing with the sodium-deficient medium (e.g. Figure 5, Table 2). In three of these experiments a low-sodium/low-chloride Krebs solution was used, when the initial diminution was less and the subsequent recovery more marked (expt. 3 in Table 2). On returning to normal Krebs solution, the potential change evoked by 5-HT resembled that seen initially and included an after-hyperpolarization. Response amplitude, however, was rarely restored fully (Figure 5f, Table 2).

The 5-HT depolarizations which persisted in sodium-deficient solutions were greatly reduced in amplitude, and in some cases abolished, when calcium ions were also omitted from the superfusion medium. The relative amplitudes of 5-HT depolarizations in 3 experiments in which ganglia were exposed sequentially to solutions deficient in sodium and deficient in sodium and calcium are given in Table 2 (1st control = 100%). Note that in sodium-free solution, removing calcium ions considerably reduced the amplitude or abolished the response (expt. 1, 2), while in the low-sodium medium (expt. 3) the reduction was less marked. Removal of $[Ca]_o$ led to some instability in the recorded baseline. In experiments 2 and 3 replacement of the calcium led to a partial recovery of the response, as did subsequent superfusion with Krebs solution in expts 1 and 2.

This apparent ability of calcium ions to support 5-HT depolarizations recalls Woolley's proposition (1958) that 5-HT might act by carrying calcium ions across the smooth muscle cell membrane. However, with ganglion cells, removal of only calcium ions from the superfusion fluid (Figure 6a) did not reduce depolarization amplitude. On the contrary, in 2 of 4 experiments (e.g. Figure 6a), there was a clear indication that the responses were of larger amplitude and area in the calcium-free medium. That enhancement of the responses had occurred was strengthened by the observation that on returning to normal Krebs solution response amplitude was reduced in all 4 experiments. In one further experiment, superfusion with calcium-rich Krebs solution reduced rather than enhanced response amplitude.

It should not be assumed that the effect of calcium ions is simply due to their presence or absence as charge carriers, for calcium ions are likely to alter membrane resistance (see Frankenhaeuser & Hodgkin, 1957; Brown, Brownstein & Scholfield, 1972; Lees & Wallis, 1974) and, thus, alter the potential resulting from the movement of other ions. It was noticeable that the high frequency noise, present to a variable degree from experiment to experiment in the baseline trace, was diminished in the presence of calcium-free media, perhaps suggesting a reduction

Table 2 Effect of removal of sodium ions and subsequent removal of calcium ions on the amplitude of 5-hydroxytryptamine depolarizations

Sequence of solutions (times indicate duration of exposure to solutions)		*Expt. 1*	*Expt. 2*	*Expt. 3*
Krebs	1st control	100%*	100%*	100%*
	2nd control	85%	84%	96%
	3rd control	81%	87%	83%
		Zero $[Na^+]_0$	Zero $[Na^+]_0$	25 mM $[Na^+]_0$
Low $[Na^+]_0$	20-30 min	41%	21%	53%
	50-100 min	64%	32%	83%
Low $[Na^+]_0$ Zero $[Ca^{2+}]_0$	10-30 min	25%	0%	44%
	40-100 min	17%	0%	52%
Low $[Na^+]_0$	10-80 min	12%	20%	82%
Krebs	40-50 min	37%	24%	60%
	60-80 min	35%	36%	54%

* Depolarization amplitude expressed as a percentage, initial depolarization in Krebs solution taken as 100, controls at 30 min intervals. Each value is a single determination or the mean of 2 determinations.

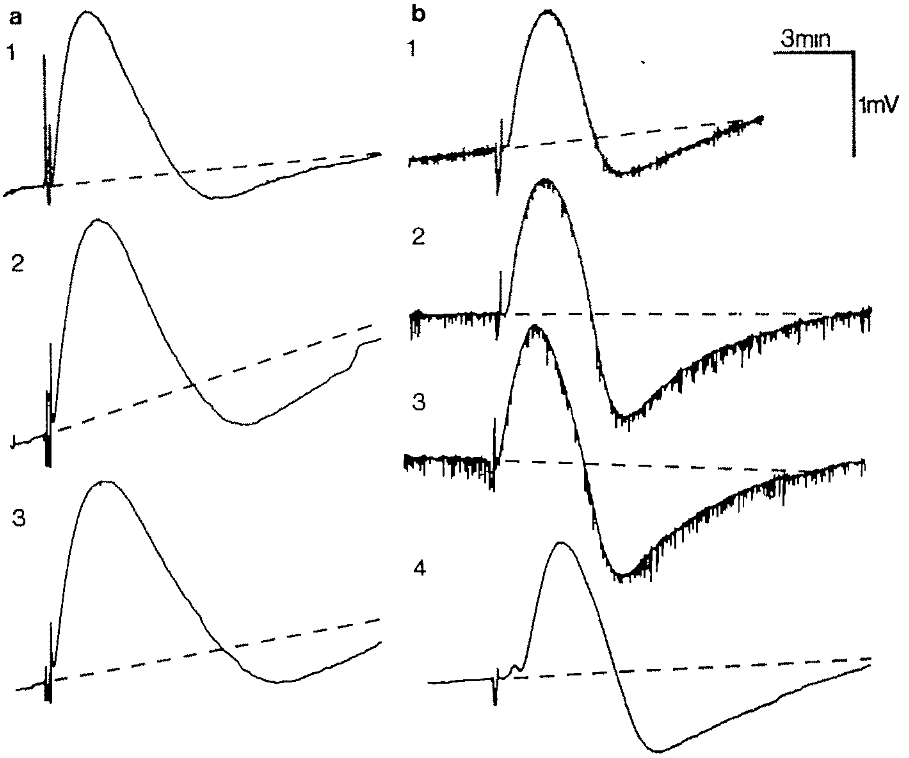

Figure 6 Effect of removing calcium ions (a) and potassium and calcium ions from the superfusion medium (b) on 5-hydroxytryptamine (5-HT) depolarizations. All responses to injection of 0 2 μmol 5-HT. (a) (1) Control, (2) 30 min and (3) 67 min in calcium-free Krebs solution. (b) Another preparation: (1) control, (2) 50 min and (3) 80 min in potassium-free Krebs solution, (4) 51 min in potassium-free/calcium-free Krebs solution.

in membrane resistance (e.g. Figure 6b). Removal of potassium ions from the superfusion fluid increased the baseline noise and usually increased the amplitude of both the depolarization and after-hyperpolarization (Figure 6b, 2 and 3). The response was maintained when the superfusion fluid was changed to one containing neither potassium nor calcium ions (Figure 6b, 4), but there was a great reduction in the high frequency noise. From the results of Figure 6b and similar experiments, it was clear that potassium ions in the external medium were not essential for either depolarization or after-hyperpolarization.

The nature of the after-hyperpolarization

The after-hyperpolarization, which follows depolarization of the superior cervical ganglion with ACh, is due to the activity of a ouabain-sensitive electrogenic sodium pump (Lees & Wallis, 1974). Characteristically, the rate of development and usually the amplitude of the after-hyperpolarization are reduced in the absence of external potassium ions. In the experiments described here, the after-hyperpolarization which followed a 5-HT depolarization was clearly increased in amplitude in 3 out of 4 experiments in potassium-free Krebs (e.g. Figure 6b). This increase was sustained even though superfusion with the potassium-free medium was prolonged. Ouabain (10 μM) initially reduced the amplitude of the 5-HT after-hyperpolarization and then abolished it (Figure 7). In one experiment, dinitrophenol (0.3 mM) also abolished the after-hyperpolarization but itself produced a prolonged depolarization of the ganglion. In sodium-deficient solutions, the after-hyperpolarizations were quickly abolished. Unlike the situation with ACh after-hyperpolarizations (Lees & Wallis, 1974), a calcium-free superfusion medium did not appear to alter greatly the amplitude of the after-hyperpolarization induced by 5-HT.

In the presence of nicotine, 5-HT induces a hyperpolarization and not a depolarization of

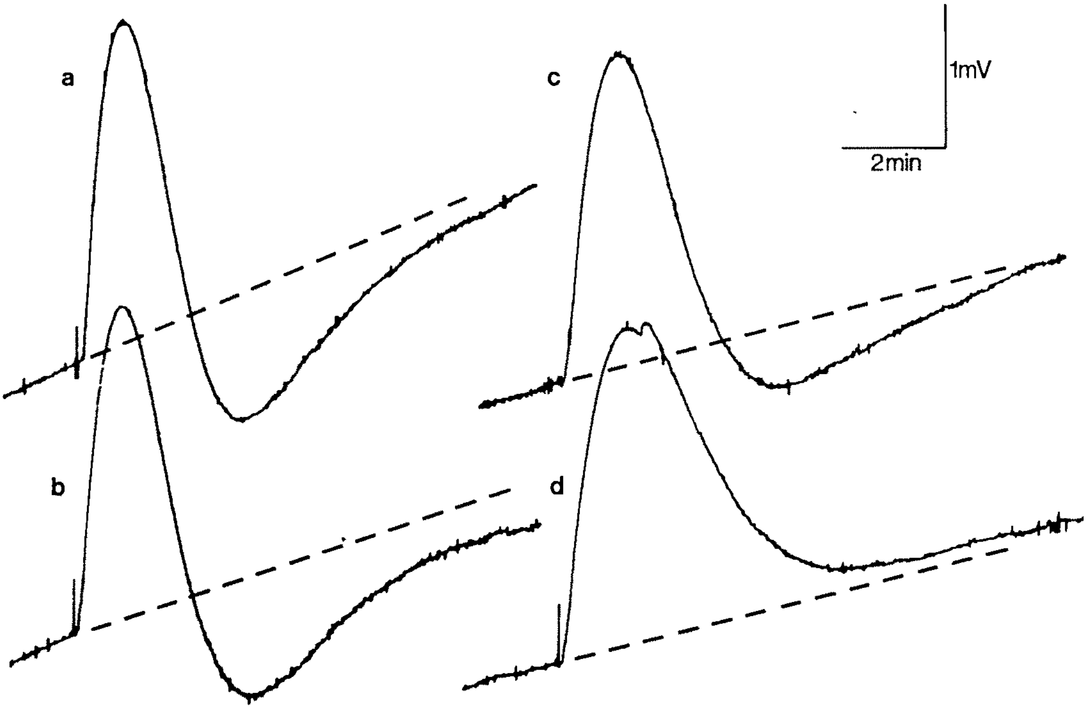

Figure 7 Effect of ouabain on 5-hydroxytryptamine (5-HT) depolarizations. (a-d) Responses to injection of 0.2 µmol 5-HT. (a) and (b) Controls, (c) 23 min and (d) 43 min after superfusion with ouabain (10 µM).

bullfrog ganglion cells (Watanabe & Koketsu, 1973). In two experiments where 5-HT evoked depolarizing responses with pronounced after-hyperpolarizations, nicotine (10 µM) depressed the amplitude of the depolarization and abolished the after-hyperpolarization of rabbit ganglia.

Possible presynaptic actions of 5-HT

If the rat superior cervical ganglion is arranged in the sucrose-gap so that the recorded potential differences arise between the proximal pole of the ganglion and the cervical sympathetic trunk, depolarizations induced on superfusion with ACh are thought to arise mainly in the preganglionic nerve terminals (Koketsu & Nishi, 1968). This experimental arrangement is shown diagrammatically in Figure 8. When injections of 5-HT were made into the superfusion stream, a depolarization was recorded (Figure 8), but the after-hyperpolarization was either absent or small in amplitude. The depolarization and after-hyperpolarization evoked by 5-HT in the same ganglion, but in the ganglion cells of the distal pole, are shown for comparison; note that the depolarization was larger and the after-hyperpolarization much more pronounced. Supporting evidence that records from the proximal pole were from structures other than ganglion cells comes from observations on the differential effects of hexamethonium. In a concentration (1.38 mM) which rapidly blocks ganglionic transmission, hexamethonium is known to potentiate the amplitude and particularly the area of 5-HT depolarizations of ganglion cells (Wallis & Woodward, 1974). Thus, in 5 experiments, ganglion cell depolarizations were 55 ± 14% (mean ± s.e. mean) larger in amplitude and 203 ± 81% (mean ± s.e. mean) larger in area in the presence of hexamethonium (1.38 mM) compared to control responses. In contrast, proximal pole responses evoked by 5-HT were not potentiated in amplitude in any of 4 experiments but reduced by 13 ± 7% (mean ± s.e. mean); their area was not significantly altered.

Discussion

An excitatory action of 5-HT on sympathetic ganglion cells detected by end-organ responses, discharge in post-ganglionic fibres or ganglion depolarization has been frequently observed

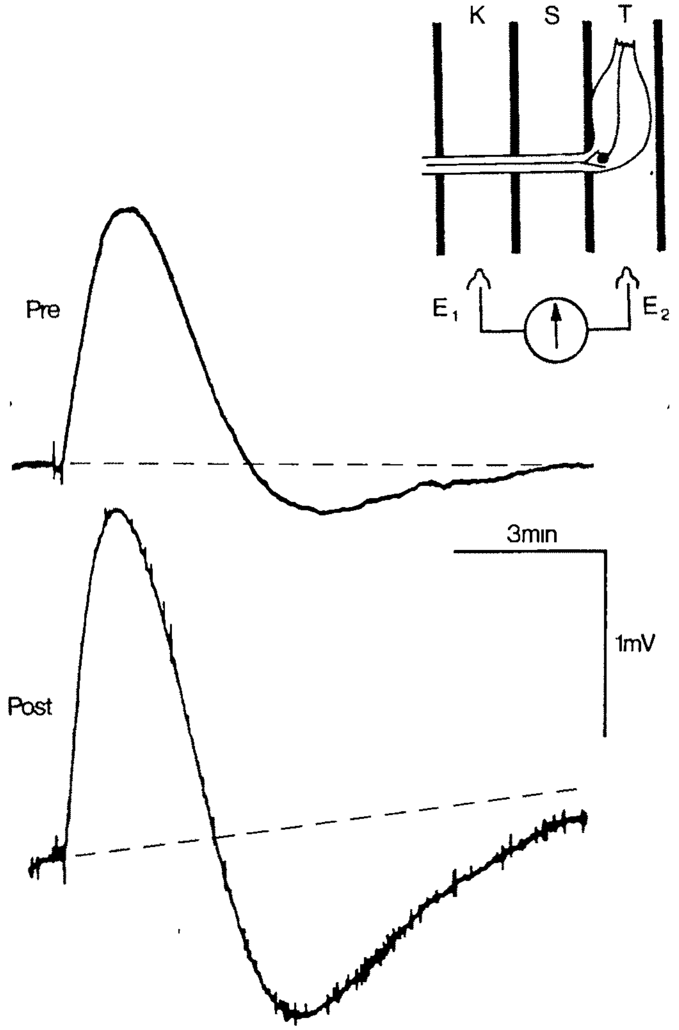

Figure 8 The effect of 5-hydroxytryptamine (5-HT) on the resting potential recorded from the proximal and distal poles of the ganglion. Inset: schematic drawing of experimental arrangement for recording from the proximal pole. The potential change is presumed to arise mainly in the preganglionic nerve terminals (see text). E_1 and E_2, calomel electrodes; K, S and T, Krebs solution, sucrose solution and test solution, respectively.
Pre: record from proximal pole of ganglion. Post: record from distal pole of ganglion with the usual recording arrangement and from the same preparation. Responses to injection of 0.2 μmol 5-HT, depolarization upwards.

(Trendelenburg, 1956, 1957; Hertzler, 1961; Gyermek & Bindler, 1962; de Groat & Volle, 1966). In our experiments, 5-HT (100 μM) induced a rapid but unsustained depolarization, substantial repolarization occurring during exposure to the amine. This repolarization probably reflects the tachyphylaxis produced by relatively high concentrations or prolonged exposures to 5-HT. Tachyphylaxis was also observed by Haefely (1974) in the cat superior cervical ganglion, but not by Watson (1970) in the same ganglion of the rat. An alternative explanation for the repolarization might be that the amine evoked a hyperpolarization of slower onset. Haefely (1974) sometimes observed small hyperpolarizations on injection of 5-HT in amounts below threshold for depolarization; Watanabe & Koketsu (1973) found that 5-HT hyperpolarized bullfrog ganglion cells when the depolarizing action of the amine on these cells was blocked by nicotine.

However, in our experiments hyperpolarizations were only seen on removal of the 5-HT or secondary to the depolarization induced by injections into the superfusion stream; nicotine in fact abolished the after-hyperpolarizations.

By injecting small amounts of 5-HT dissolved in less than 1 ml of Krebs solution into the superfusion stream a series of depolarizing responses, relatively constant in magnitude, could be obtained or an ED_{50} value established. The quantities injected are not dissimilar to those employed for close arterial injection to the cat superior cervical ganglion by various authors, e.g. 0.01-0.1 μmol (de Groat & Volle, 1966), 0.002-0.025 μmol (Haefely, 1974), 0.74-1.23 μmol (Machova & Boska, 1969). In the cat inferior mesenteric ganglion, modification of the discharge frequency was induced by close arterial injection of 0.003-0.025 μmol (Gyermek & Bindler, 1962). Submaximal depolarizations evoked by injections of 5-HT, choline or ACh were similar in time course, but only those to 5-HT were consistently followed by an after-hyperpolarization. ACh (in the presence of physostigmine) and choline are known to evoke after-hyperpolarizations (Lees & Wallis, 1974), but continuous superfusion with the depolarizing agent for several minutes is necessary to induce this response. Monoamine oxidase inhibitors had very little effect on the responses to 5-HT, apart from causing some increase in their duration. This suggests that enzymatic destruction is not greatly affecting the amount of amine reaching the receptors and is in accord with the finding that monoamine oxidase inhibitors do not potentiate the action of 5-HT on the rat stomach strip (Vane, 1959).

The dependence of 5-HT depolarizations on external sodium ions suggests that the principal permeability change involved is an increase in sodium ion conductance (G_{Na}). The depolarizations were initially depressed but subsequently recovered to some extent in sodium-deficient solutions and the residual depolarization appeared to be dependent on calcium ions. Thus, 5-HT may induce a concomitant increase in G_{Ca}. Depolarizations to ACh are readily abolished in sodium-deficient solutions (Wallis & Woodward, unpublished observations), so that it appears that calcium ions may substitute for sodium ions as charge carriers during depolarization more readily in the case of responses to 5-HT than in the case of responses to ACh. Nevertheless, calcium ions can support an ACh-induced depolarization of the cat superior cervical ganglion at least (Pappano & Volle, 1966). In the rabbit ganglion, any depolarization resulting from entry of calcium did not make any appreciable contribution to the total depolarization induced by 5-HT when the extracellular sodium concentration was normal, for omitting calcium ions alone from the superfusion medium did not diminish the amplitude of the depolarization.

Alternative ionic mechanisms which might be considered include: (i) a reduced G_K (as suggested by Jéquier (1965) for 5-HT restoration of transmission and by Weight & Votava (1970) as the basis of the slow muscarinic depolarizing potential in frog ganglion cells); and (ii) an increased G_{Cl} (suggested as mediating GABA-induced depolarization of ganglion cells: Adams & Brown, 1973). In the absence of membrane resistance measurements, these cannot be definitely rejected, but appear unlikely for the following reasons: (i) the 5-HT depolarization persists in chloride-free solution, unlike that produced by GABA; (ii) the 5-HT depolarization is enhanced during DMPP-induced hyperpolarization (Jaramillo & Volle, 1968), whereas (assuming E_K is unchanged) it should be reduced if mediated by a decrease in G_K since the electrochemical gradient and net passive efflux of K^+ ions will be reduced (cf. Weight & Votava, 1970). In our experiments, 5-HT depolarization was sometimes increased in potassium-free solution (e.g. see Figure 6), but although this is compatible with a fall in G_K it is inconclusive. It may be noted that, in some snail neurones, 5-HT depolarization results from an increase in membrane conductance, probably of G_{Na} (Gerschenfeld & Stefani, 1968). However, depolarization and hyperpolarization as a result of reduced membrane conductances have also been reported (Paupardin-Tritsch & Gerschenfeld, 1973).

The after-hyperpolarization which followed a 5-HT-induced depolarization has also been observed in cat ganglia and some preparations also display a subsequent late depolarization (Machova & Boska, 1969; Haefely, 1974); we did not observe any late depolarization in our experiments. ACh after-hyperpolarizations are probably a consequence of active extrusion of sodium ions by an electrogenic pump (Lees & Wallis, 1974) and Watanabe & Koketsu (1973) have suggested that the hyperpolarization evoked by 5-HT in bullfrog sympathetic ganglia is also due to activation of an electrogenic sodium pump. That the after-hyperpolarization evoked by 5-HT is generated by electrogenic pumping of sodium ions is consistent with its abolition in sodium-deficient solutions and by ouabain and dinitrophenol, but not with the increase in amplitude in potassium-free solutions. Extracellular potassium ions are known to be necessary for activation of the pump (Lees & Wallis, 1974). Further, in contrast to the situation with after-hyperpolarizations evoked by 5-HT, calcium-free solutions reduce or abolish ACh after-hyperpolarizations. These discrepancies may indicate that 5-HT induces conductance changes which outlast the phase of depolarization.

The depolarizations recorded from the proximal pole of the ganglion may tentatively be ascribed to the presynaptic terminals for two reasons. First, there are normally no or very few ganglion cells demonstrable electrophysiologically whose axons or axon collaterals project caudally along the cervical sympathetic trunk and, secondly, the 'presynaptic' depolarizations showed some differences from 5-HT-induced depolarization of ganglion cells, e.g. reduced or absent after-hyperpolarizations and no potentiation by hexamethonium.

This work was supported by a grant from the Medical Research Council. We are indebted to Sandoz Products Ltd. for the gift of 5-HT, May & Baker Ltd. for the gift of hexamethonium and LR Industries Ltd. for the gift of rubber condoms.

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(Received April 1, 1975.
Revised June 12, 1975.)

Br. J. Pharmac. (1975), 55, 213-219

PARALLEL BIOASSAY OF BOMBESIN AND LITORIN, A BOMBESIN-LIKE PEPTIDE FROM THE SKIN OF *Litoria aurea*

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1 The spectrum of biological activity exhibited by litorin, a bombesin-like nonapeptide found in extracts of the skin of the Australian leptodactylid frog *Litoria aurea* was compared with that exhibited by the tetradecapeptide bombesin.

2 Litorin proved to be more potent than bombesin on isolated smooth muscle preparations and on the urinary bladder *in situ*. However, it was less potent on dog systemic blood pressure and kidney vasculature, activation of the renin-angiotensin system being slight or lacking.

3 Gastrin release and acid secretion produced by litorin was more rapid in onset but less intense and less sustained than that elicited by bombesin. The same could be observed for pancreatic secretion.

4 Gall bladder contraction stimulated by litorin was probably caused by a double action of the peptide, directly on the bladder smooth muscle, and indirectly by cholecystokinin release.

5 In its effects on the myo-electric activity of the dog duodenum (inhibition of spikes and increase in frequency of pacesetter potentials leading to the appearance of a sequence of slow and small potentials) litorin possessed approximately 50 to 70% of the activity of bombesin.

Introduction

After the isolation of bombesin from methanol extracts of the skin of the two European discoglossid frogs *Bombina bombina* and *Bombina variegata variegata* and of alytesin from skin extracts of another European discoglossid frog, *Alytes obstetricans* (Anastasi, Erspamer & Bucci, 1971), a third bombesin-like peptide was isolated by our research group from methanol extracts of the skin of the Australian leptodactylid frog *Litoria (Hyla) aurea*. It is presumably present in the skin of other *Litoria* species and of other Australian amphibian genera as well (Anastasi, Erspamer & Endean, 1973; Erspamer, Negri, Falconieri Erspamer & Endean, 1975). Litorin has been reproduced by synthesis (Angelucci & De Castiglione, 1975). Its amino acid composition and sequence are shown below, together with those of bombesin and alytesin:

Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-
His-Leu-Met-NH_2 bombesin
Pyr-Gly-Arg-Leu-Gly-Thr-Gln-Trp-Ala-Val-Gly-
His-Leu-Met-NH_2 alytesin
Pyr————————Gln-Trp-Ala-Val-Gly-
His-Phe-Met-NH_2 litorin.

It may be seen that whereas only minor differences exist between the tetradecapeptides bombesin and alytesin, the latter being Gly^2-Thr^6-bombesin, litorin differs conspicuously from the other two members of the family, being a simple nonapeptide, lacking the entire amino acid sequence Gln-Arg-Leu-Gly-Asn and having a phenylalanine residue substituted for the leucine residue at position 2 from the C-terminus.

This paper describes the results of a parallel bioassay of bombesin and litorin on a number of *in vitro* and *in vivo* test objects, virtually covering the whole spectrum of biological activity of bombesin. It will be seen that litorin closely mimics bombesin in its pharmacological effects although there are some notable differences.

Methods

The methods employed were identical to those described in detail in earlier papers (Erspamer, Falconieri Erspamer, Inselvini & Negri, 1972a;

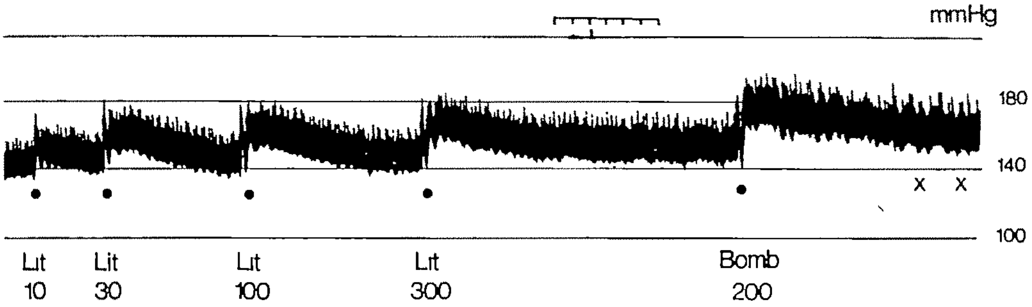

Figure 1 Blood pressure of a dog anaesthetized with sodium pentobarbitone (40 mg/kg, i v.). Time marks, 1 minute. The effects of increasing intravenous doses (ng/kg) of litorin (Lit) and of one dose of bombesin (Bomb) are shown. Note the moderate dose-response relationship for litorin, especially in the duration of pressure rise. Bombesin was more potent than litorin both in intensity and duration of action. At X drum stopped for 10 minutes.

Erspamer, Melchiorri & Sopranzi, 1972b; Bertaccini, Erspamer & Impicciatore, 1973; Bertaccini, Erspamer, Melchiorri & Sopranzi, 1974; Erspamer, Improta, Melchiorri & Sopranzi, 1974).

To measure the flow of gall bladder bile, and hence indirectly gall bladder contraction, dogs provided with a Gregory cannula implanted in the duodenum behind the orifice of the choledochus, were injected intravenously 8 h before the experiment, with a total dose of 50 μCi of ^{125}I-biligraphin. Bile was collected from the cannula and the radioactivity of the bile was counted in a well type scintillator (Italelettronica S.p.A., Rome). During the interval between biligraphin administration and the start of the experiment, all biligraphin had been eliminated except that stored in the gall bladder and thus only gall bladder bile was labelled.

The samples of bombesin and litorin used were prepared by synthesis at the Farmitalia S.p.A. Research Laboratories, Milan; ^{125}I-biligraphin was obtained from Schering A.G., Berlin.

Results

Isolated smooth muscle preparations

Table 1 shows the activity of litorin on various smooth muscle preparations expressed as a percentage of that of bombesin (taken as 100). On the urinary bladder *in situ* of the rat and the guinea-pig, the activity of litorin was 100-300 (3 preparations), and 100-200 (3 preparations), respectively. On all these preparations bombesin produced stimulation, involving the appearance and reinforcement of movements and increase of tone.

In general the response to litorin in isolated preparations was more rapid in onset than that to bombesin and, upon washing with fresh physiological solution, relaxation was more rapid. The same rapid onset and disappearance of the activity of litorin could also be clearly seen in the rat urinary bladder *in situ* Tachyphylaxis frequently occurred, but it was less intense for litorin than for bombesin.

Systemic blood pressure

Owing to the rather rapid appearance of tachyphylaxis, data on blood pressure can only be approximate.

Dog Like bombesin, litorin elicited a moderate, prolonged rise in systemic blood pressure, rarely exceeding 30 to 40 mmHg. The threshold dose by rapid intravenous injection was of the order of 10-30 ng/kg. The effect showed little relationship to dose in its intensity although a relationship between dose and duration of effect was more evident. Bombesin was more active than litorin, especially in the duration of the pressure rise (Figure 1).

Table 1 Activity of litorin expressed as a percentage of that of bombesin (taken as 100) on isolated smooth muscle preparations

Smooth muscle	*No. of preparations*	*Activity of litorin*
Rat uterus	10	200-600
colon	5	300-800
stomach	4	200-300
urinary bladder	8	150-500
Guinea-pig large intestine	6	200-300
urinary bladder	7	150-250
gall bladder	5	50-100
Kitten small intestine	20	150-350

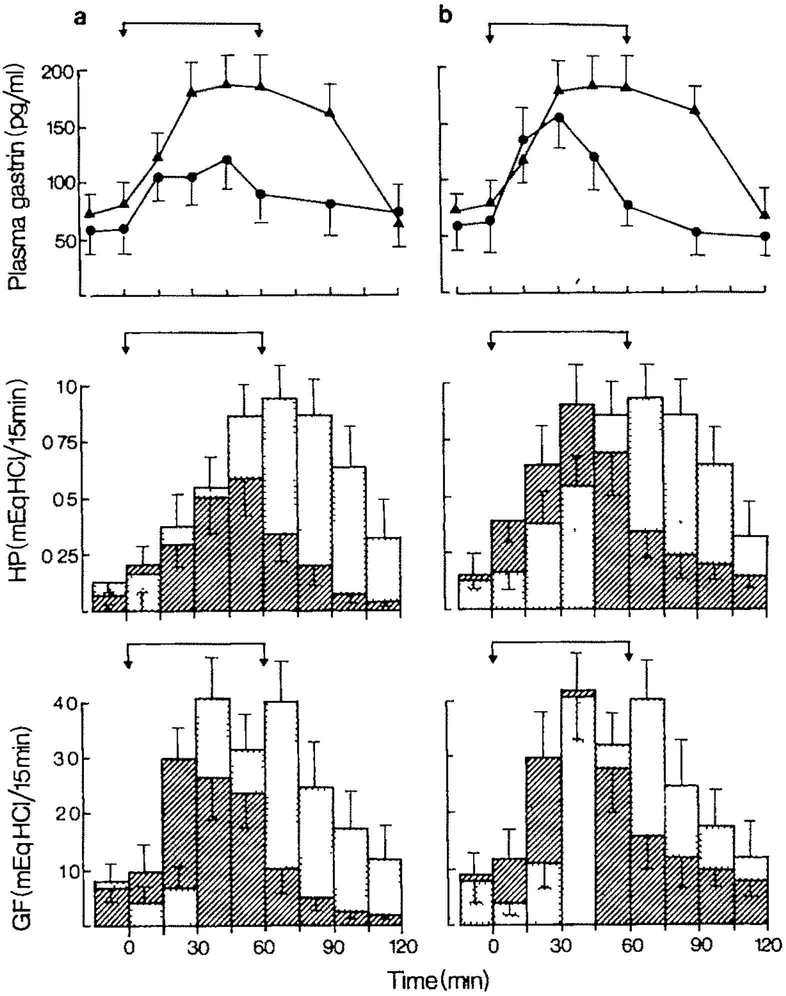

Figure 2 Gastric fistula dogs provided with Heidenhain pouches. Plasma gastrin levels and acid output, in the Heidenhain pouch (HP) and in the main stomach (GF), following intravenous infusions, for 60 min, of bombesin (▲ and stippled areas) and of litorin (● and hatched areas). Doses of bombesin were 10 ng kg^{-1} min^{-1} both in (a) and (b), doses of litorin were 10 ng kg^{-1} min^{-1} in (a) and 20 ng kg^{-1} min^{-1} in (b) Columns represent the mean of 3 measurements in each of 3 dogs. Vertical bars show s.e. mean.

Rabbit In this species litorin was generally hypotensive at 10 to 100 ng/kg doses, given intravenously. Higher doses produced unpredictable results (hypertension followed by hypotension or *vice versa*), with evident tachyphylaxis. Comparison with bombesin was impossible.

Rat The rapid hypertensive effect of litorin (threshold 3-10 ng/kg, by rapid i.v. injection) was not sustained and was sometimes followed by a fall of blood pressure. Tachyphylaxis was evident, especially at high dose levels. Bombesin was often, but not always, less potent than litorin, but its effect was more prolonged.

Gastrin release and acid gastric secretion

The effects of intravenous infusions of 10 and 20 ng kg^{-1} min^{-1} of litorin were compared, in dogs provided with gastric fistulae and Heidenhain pouches, with the effects of 10 ng kg^{-1} min^{-1} of bombesin. Results are shown in Figure 2.

It may be seen that litorin was less effective than bombesin in releasing gastrin and stimulating

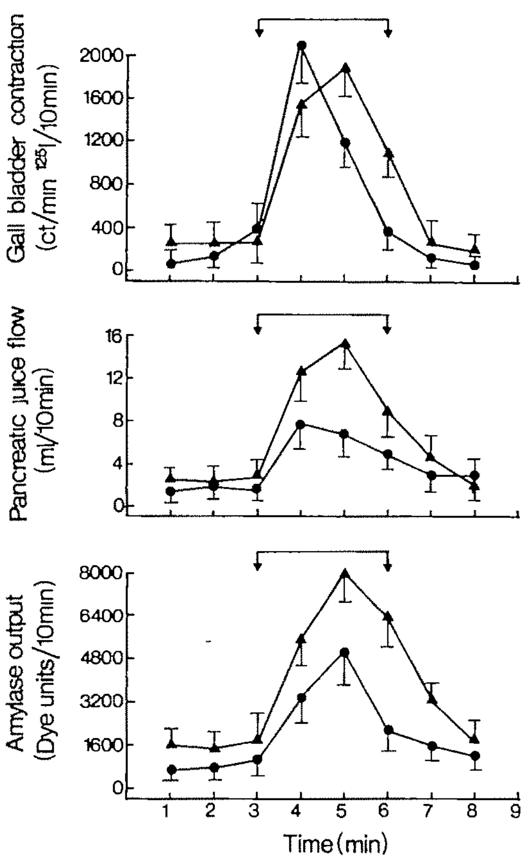

Figure 3 Conscious dogs provided with chronically implanted cannulae for collection of bile and pancreatic juice. Gall bladder contraction (as inferred from flow of radioactive bile), pancreatic juice flow and amylase output following intravenous infusion, for 60 min, of 10 ng kg^{-1} min^{-1} of bombesin (▲) and litorin (●) Each point represents the mean of 2 measurements in each of 2 dogs. Vertical bars show s.e. mean.

gastric acid secretion, both in the main stomach and in the Heidenhain pouch. In producing a maximum response, litorin was approximately half as active as bombesin on a weight basis, and one third as active on a molar basis. However, responses to litorin were more rapid and after having reached their maximum, less sustained, declining even during the infusion period. Fifteen min after the infusion of litorin 20 ng kg^{-1} min^{-1} had been stopped, gastrin levels had returned to basal values and gastric acid output was similarly greatly reduced. This is in sharp contrast to bombesin, the maximum effects of which persisted unchanged for 15-30 min after the infusion had been discontinued.

The threshold dose of litorin affecting gastrin release was 5 ng kg^{-1} min^{-1}. Plasma levels of gastrin rose from 66 ± 18 pg/ml to 81 ± 17 pg/ml.

Pancreatic secretion

Like bombesin, litorin produced in the dog the secretion of a pancreatic juice rich in amylase. However, the effect of 10 ng kg^{-1} min^{-1} litorin, infused over a 30 min period, was considerably less intense than that produced by the same dose of bombesin. Moreover, pancreatic response declined earlier for litorin than for bombesin, during the infusion period (Figure 3).

Gall bladder

Dog The motility of the gall bladder, as recorded in anaesthetized animals by measuring the bile flow through a duodenal cannula, was stimulated by bombesin and litorin as shown in Figure 3.

In this preparation litorin was as potent as bombesin, but its effects were more rapid in onset and disappearance. A peak effect with litorin was obtained after 10 min and with bombesin after 20 minutes. At the end of the 30 min infusion period, the gall bladder tone had returned to pre-infusion levels in the case of litorin, while remaining elevated for bombesin.

Guinea-pig Rapid intravenous injection of litorin caused a prompt contraction of the gall bladder, followed by rapid relaxation. The threshold dose was of the order of 2-10 ng/kg and there was often a definite dose-response relationship. Litorin was 2 to 3 times more potent than bombesin in the intensity of the response produced, on a weight basis, but equal to bombesin in the duration of the response produced.

It should be noted that the action of litorin on the guinea-pig gall bladder may well be compared with caerulein, the most potent stimulant so far known. In fact, in 14 experiments the effect of litorin was 5 to 20% that of caerulein (Figure 4).

Myo-electric activity of the gut

Caprilli, Melchiorri, Improta, Vernia & Frieri (1974) have shown that bombesin is capable of abolishing spikes and of increasing the frequency of pacesetter potentials in the duodenal and jejunal musculature of conscious dogs, which leads to disappearance of rhythmicity and appearance of an irregular sequence of slow and small potentials (uncoupling of local oscillators). Litorin had a similar effect (Figure 5). As in the case of bombesin, disappearance of spikes elicited by litorin was accompanied by complete arrest of

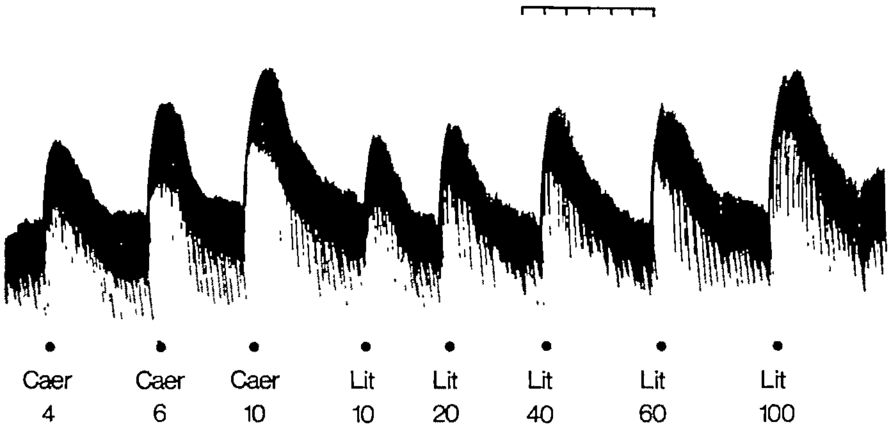

Figure 4 Guinea-pig anaesthetized with urethane. Responses of the gall bladder *in situ* to different intravenous doses (ng/kg) of caerulein (Caer) and litorin (Lit) are shown. Time marks, 1 minute. Although responses to caerulein and litorin were very similar increases in response produced by increasing the dose were more striking for caerulein than for litorin.

mechanical activity of the upper small intestine. The secondary hypermotility which followed the interruption of the peptide infusion was more evident for litorin than for bombesin.

Kidney function and renin release

In the dog bombesin caused a striking antidiuretic effect due to reduction of the glomerular filtration rate caused by a fall in intraglomerular hydrostatic pressure. This, in its turn, was provoked by vasoconstriction, invariably leading also to an activation of the renin angiotensin system.

Litorin was considerably less active than bombesin on the kidney. In fact, following intravenous infusion over a 20 min period of as much as 20 ng kg^{-1} min^{-1} of litorin, the glomerular filtration rate fell (not significantly) from 115 ± 25 to 76 ± 11 ml/min, and renin activity in arterial blood remained unchanged (1.9 ng/ml in control-blood and 2.2 ng/ml in litorin-blood). As bombesin produced significant effects on these parameters at infusion rates ranging between 1 and 3 ng kg^{-1} min^{-1} it is evident that, on a weight basis, litorin was at least 7 to 20 times less potent than bombesin.

Discussion

The nonapeptide bombesin is the smallest bombesin-like peptide found in amphibian skin. It differs from the C-terminal nonapeptide of the tetradecapeptide bombesin in having the pyroglutamyl^1 and the phenylalanyl^8 residues substituted for the threonyl^1 and the leucyl^8 residues, respectively.

It will be shown in a forthcoming paper that the activity spectrum of the C-terminal nonapeptide of bombesin closely resembles that of bombesin, with only minor quantitative differences. Litorin, on the other hand, is definitely, although not markedly different from bombesin.

Apparently the most striking diversity lies in the fact that the actions of litorin are more rapid in onset and in disappearance than those of bombesin, both in *in vitro* and *in vivo*. This could signify that it occupies its receptor sites in the target cells more rapidly but less tenaciously than bombesin. It is probable that the rapidity of action accounts for the more intense effects on isolated smooth muscle preparations and on the gall bladder *in situ*; the less tenacious binding to the receptors may account for the less intense and the less prolonged effects on blood pressure, on intrarenal vessels, on electrical activity of the gut, and finally on release of gastrin and cholecystokinin.

The gastrin-releasing activity of litorin is unequivocally demonstrated by the increase in plasma levels of immunoreactive gastrin, observed during the infusion of the peptide. However, cholecystokinin release is only presumptive, although strongly supported by the effects of litorin on pancreatic secretion and on gall bladder motility.

It seems probable that contraction of the gall

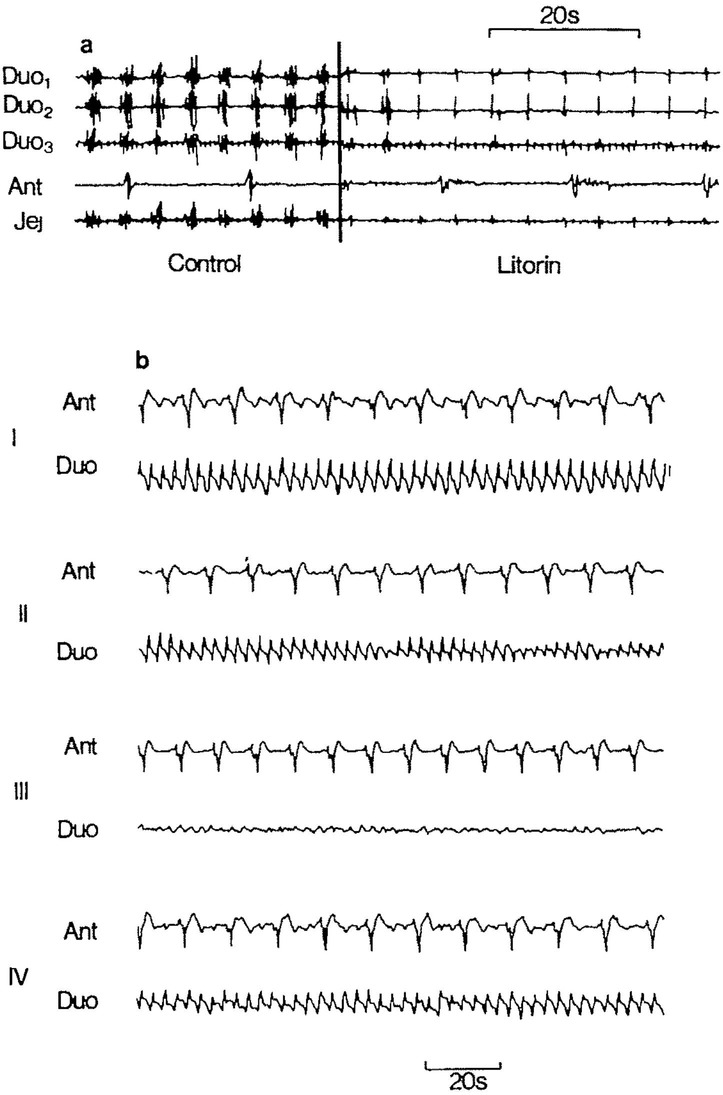

Figure 5 Conscious dog provided with electrodes chronically implanted on the serosal surface of different gastrointestinal segments. (a) Fast activity (spikes) evoked by a meal in the 1st, 2nd and 3rd duodenal portion (Duo$_1$, Duo$_2$, Duo$_3$), the antrum (Ant) and the jejunum (Jej), before (Control) and 10 min after start of an intravenous infusion of 2 ng kg^{-1} min^{-1} of litorin (Litorin). (b) Pacesetter potentials of the antrum (Ant) and the duodenum (Duo) before litorin administration (I), during an intravenous infusion of 10 ng kg^{-1} min^{-1} of litorin (II and III, at 10 and 30 min of infusion, respectively), and 20 min after discontinuing litorin infusion (IV).

bladder *in vivo* is the result of a twofold action of litorin, one direct on the bladder smooth muscle, and the other indirect, secondary to release of cholecystokinin. Evidence in favour of a direct mechanism is provided by the rapidity in onset and disappearance of litorin stimulation, particularly well shown by the gall bladder of the guinea-pig, as well as by the dissociation between activity ratios of litorin to bombesin on gall bladder and on pancreas. Although litorin was as active as bombesin on gall bladder motility in the dog, it was much less active on pancreatic secretion. Only measurements of cholecystokinin levels in plasma following bombesin and litorin administration will elucidate the relative importance of the two mechanisms of action.

The most striking quantitative difference in activity between litorin and bombesin probably lies in the renal effect of the peptides. It appears that litorin has very little action on the afferent glomerular arteries.

The possibility cannot be excluded that the less sustained effects of litorin *in vivo* stem partially from the fact that, in contrast to bombesin, litorin lacks renal actions. As a consequence, both gut hormones released by litorin and litorin itself could be more easily excreted and/or inactivated by the kidney.

Litorin offers a natural example of how relatively small changes in the amino acid composition of a peptide may bring about considerable changes in its biological activity. Synthesis of bombesin and litorin analogues seem to offer interesting possibilities.

This work was supported by grants from the Consiglio Nazionale delle Ricerche, Rome.

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(Received April 10, 1975.
Revised May 27, 1975.)

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RELATIVE POTENCY OF BOMBESIN-LIKE PEPTIDES

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1 The pharmacological activity of two natural bombesin-like peptides, alytesin and litorin, and 25 related synthetic peptides has been compared to that of bombesin.

2 The minimum length of the amino acid chain required for the first appearance of bombesin-like effects was represented by the C-terminal heptapeptide, and the minimum length for maximal effects by the C-terminal nonapeptide. The latter possessed approximately the same activity as bombesin and may be considered a good substitute.

3 Both the tryptophan and histidine residues seemed to be essential for bombesin-like activity.

4 The C-terminal octapeptide was less active than either bombesin or the C-terminal nonapeptide and its action was more rapid in onset and less sustained.

5 Litorin apparently has an intermediate position between bombesin octapeptide and bombesin nonapeptide in the speed and duration of its effects. The relationship between structure and activity is discussed.

Introduction

Four natural bombesin-like peptides have been isolated from methanol extracts of the amphibian skin: the tetradecapeptides bombesin from *Bombina bombina* and *Bombina variegata variegata* and alytesin from *Alytes obstetricans* (Anastasi, Erspamer & Bucci, 1971), the endecapeptide ranatesin from *Rana pipiens* (Nakajima, Tanimura & Pisano, 1970), and the nonapeptide litorin from *Litoria (Hyla) aurea* (Anastasi, Erspamer & Endean, 1975). All these peptides have in common the C-terminal octapeptide, with the only difference that in bombesin and alytesin the amino acid residue at position 2 from the C-terminus is leucine, and in ranatesin and litorin it is phenylalanine.

In order to determine the minimum length of the amino acid chain required for the first appearance of bombesin activity, and the minimum length necessary for maximal effects, 25 bombesin-like peptides have been synthesized and compared with bombesin. Alytesin and litorin have been included in this comparison but ranatensin was not available. For a few peptides, and especially for the C-terminal nonapeptide of bombesin, not only were effects on smooth muscle preparations investigated, but also effects on blood pressure, gastric acid secretion, and intestinal myo-electric activity.

Methods

The methods employed were identical to those described in detail in earlier papers (Erspamer, Falconieri Erspamer, Inselvini & Negri, 1972a; Erspamer, Melchiorri & Sopranzi, 1972b, Bertaccini, Erspamer, Melchiorri & Sopranzi, 1974; Caprilli, Melchiorri, Improta, Vernia & Frieri, 1975),

Alytesin was the natural peptide prepared by Anastasi *et al.* (1971); all other peptides, including caerulein, were synthesized at the Farmitalia S.p.A., Research Laboratories, Milan.

Results

Isolated smooth muscle preparations

In the six isolated smooth muscle preparations examined in this study, only stimulant actions and

Table 1 Relative potency of bombesin-like peptides, on a weight basis (bombesin = 100)

Peptide	*Test preparations* *Rat uterus*	*Rat urinary bladder*	*Guinea-pig urinary bladder*	*Guinea-pig large intestine*	*Kitten small intestine*	*Rat large intestine*
Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$ Bombesin	100	100	100	100	100	100
Pyr-Gly-Arg-Leu-Gly-Thr-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$ Alytesin	70-100 (5)	80-120 (3)	—	75-125 (3)	70-200 (7)	75-100 (4)
Pyr-Gln-Trp-Ala-Val-Gly-His-Phe-Leu-Met-NH$_2$ Litorin	200-600 (10)	150-500 (8)	150-250 (7)	200-500 (6)	150-350 (20)	300-800 (5)
His-Leu-Met-HN$_2$	< 0.1 (2)	< 0.1 (3)	—	< 0.1 (2)	0.2 (2)	—
Ala-Val-Gly-His-Leu-Met-HN$_2$	< 0.05 (5)	< 0.01 (2)	0.1-0.2 (2)	< 0.1 (7)	< 0.1 (5)	< 0.1 (3)
BOC-Ala-Val-Gly-His-Leu-Met-NH$_2$	< 0.1 (3)	< 0.1 (3)	—	< 0.1 (3)	< 0.1 (3)	< 0.1 (3)
Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$	2.5-10 (4)	0.2-0.5 (4)	0.2-0.4 (3)	0.1-0.5 (7)	< 0.1-3 (10)	0.5-1 (3)
BOC-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$	30-60 (7)	6-14 (3)	1-3 (3)	1-3 (10)	0.5-1 (10)	3-6 (3)
Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$	20-30 (6)	2-10 (4)	2-4 (4)	2-10 (7)	1-4 (17)	10-20 (3)
BOC-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$	40-75 (9)	30-60 (4)	—	25-60 (10)	40-100 (6)	50-80 (3)
Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$	130-300 (7)	150-300 (4)	200-350 (3)	100-120 (7)	100-150 (11)	90-130 (3)
BOC-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$	160-500 (7)	300-500 (3)	—	150-180 (3)	110-160 (7)	100-130 (3)
Thr-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$	150-400 (5)	150-350 (3)	—	100-140 (7)	100-150 (5)	—
Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$	120-200 (5)	100-150 (3)	150-200 (3)	110-200 (5)	90-200 (8)	70-90 (3)
BOC-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$	30-60 (5)	25-40 (3)	—	30-60 (4)	30-110 (7)	—
Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$	50-200 (8)	100-170 (5)	200-400 (5)	100-160 (6)	80-150 (8)	90-100 (3)
BOC-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-NH$_2$	35-130 (5)	70-120 (3)	—	120-210 (5)	60-120 (6)	35-80 (3)
Asn-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$	70-100 (5)	4-10 (3)	4-7 (3)	3-12 (5)	1-4 (5)	20-40 (3)
Phe-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$	150-220 (7)	6-20 (3)	1-2 (3)	7-35 (6)	4-13 (5)	60-120 (3)
BOC-Phe-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$	250-350 (3)	20-60 (3)	—	5-25 (3)	1.5-5 (4)	—
Val-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$	65-85 (5)	1-5 (3)	1-2 (3)	2-8 (4)	1.5-5 (6)	35-70 (3)
BOC-Val-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$	200-320 (4)	2-8 (6)	—	—	5-10 (7)	—
Tyr-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$	150-250 (3)	150-200 (3)	—	80-120 (3)	100-150 (3)	—
BOC-Asn-Gln-Trp-Ala-Val-Gly-His(DNP)-Leu-Met-NH$_2$	5-12 (3)	6-13 (3)	—	1-2 (3)	1-1.5 (3)	4-6 (3)
Pyr-Gly-Arg-Leu-Gly-Thr-Gln-Trp	< 0.01 (2)	< 0.1 (2)	—	< 0.1 (2)	< 0.1 (2)	< 0.1 (2)
Pyr-Gly-Arg	< 0.01 (2)	< 0.1 (2)	—	< 0.01 (2)	< 0.1 (2)	< 0.1 (2)

Pyr, pyroglutamic acid, BOC, *ter*-butyloxycarbonyl; DNP, 2,4-dinitrophenyl; —, not tested. The number of experiments is given in parentheses.

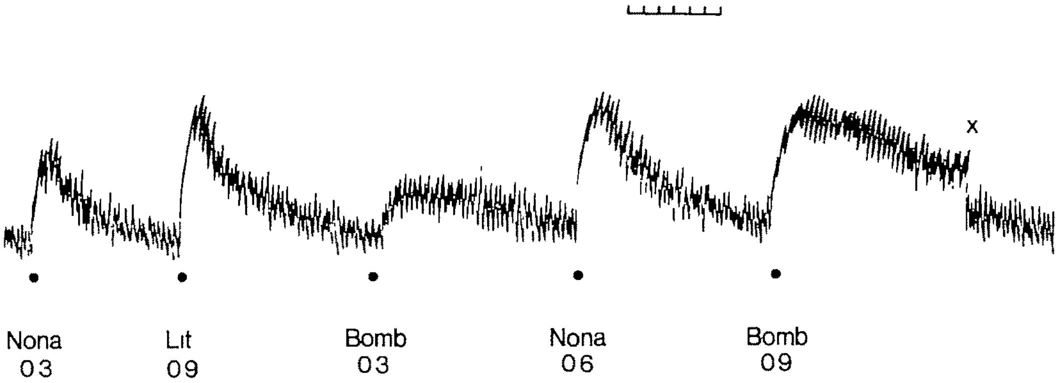

Figure 1 Rat anaesthetized with urethane. Responses of the urinary bladder *in situ* to different doses of bombesin (Bomb), litorin (Lit) and bombesin nonapeptide (Nona). Time marks, 1 minute. At x, drum stopped for 10 minutes. Duration of effect was greatest for bombesin, least for litorin.

never inhibitory effects were observed. Results are summarized in Table 1 in which the activity of bombesin was taken as 100, and that of the bombesin-like peptides was expressed as a percentage.

The stimulant effects displayed on movements and tone by all members possessing a sequence of more than eight amino acid residues were (with the exception of litorin) almost indistinguishable from those of bombesin. However, litorin and to a greater extent the octapeptides frequently caused a stimulation which was more rapid in onset and in disappearance, on washing with fresh physiological solution, than that produced by bombesin.

Results shown in Table 1 were essentially confirmed on isolated preparations of human gastrointestinal tract by Bertaccini, Impicciatore, Molina & Zappia (1974): bombesin 100, C-terminal nonapeptide 100-120, octapeptide 10-30; heptapeptide 1-5, hexapeptide < 1.

Rat and guinea-pig urinary bladder in situ

Relative potencies (bombesin = 100) on the rat and guinea-pig urinary bladder *in situ* are shown in Table 2. The figures in the table refer only to the intensity of action. For the duration of response which they produced the peptides may be ranged in the following order: bombesin = endecapeptide and decapeptide > nonapeptide > litorin > octapeptide and Asn^1-octapeptide > Phe^1-octapeptide and Val^1-octapeptide (Figure 1).

Guinea-pig gall bladder in situ

Bombesin, litorin and the bombesin nonapeptide were potent stimulants of the guinea-pig gall bladder *in situ*. In the experiment illustrated in Figure 2 the relative potency of the three above peptides and of caerulein was as follows: bombesin 100; litorin 200-250; nonapeptide 100-125; caerulein 2000.

Whereas the shapes of the contractions elicited by litorin, bombesin nonapeptide and caerulein were very similar, the contraction produced by bombesin was less rapid in onset and more sustained.

Systemic blood pressure

The relative potency of bombesin-like peptides on the blood pressure could be evaluated only approximately because of the development of tachyphylaxis.

Table 2 Potencies of bombesin-like peptides relative to bombesin (100) on the rat and guinea-pig urinary bladder *in situ*

Peptide	*Urinary bladder* in situ *Rat*	*Urinary bladder* in situ *Guinea-pig*
C-terminal		
heptapeptide	0.1 (3)	0.2 (3)
octapeptide	2.5 (3)	5-6 (3)
Asn^1-octapeptide	4.0 (3)	
Phe^1-octapeptide	2.0 (3)	
Val^1-octapeptide	1.2 (3)	
nonapeptide	100-200 (5)	150-250 (3)
decapeptide	75 (3)	100-150 (3)
endecapeptide	75 (3)	100-150 (3)
Litorin	100-200 (5)	100-200 (3)

Number of preparations given in parentheses.

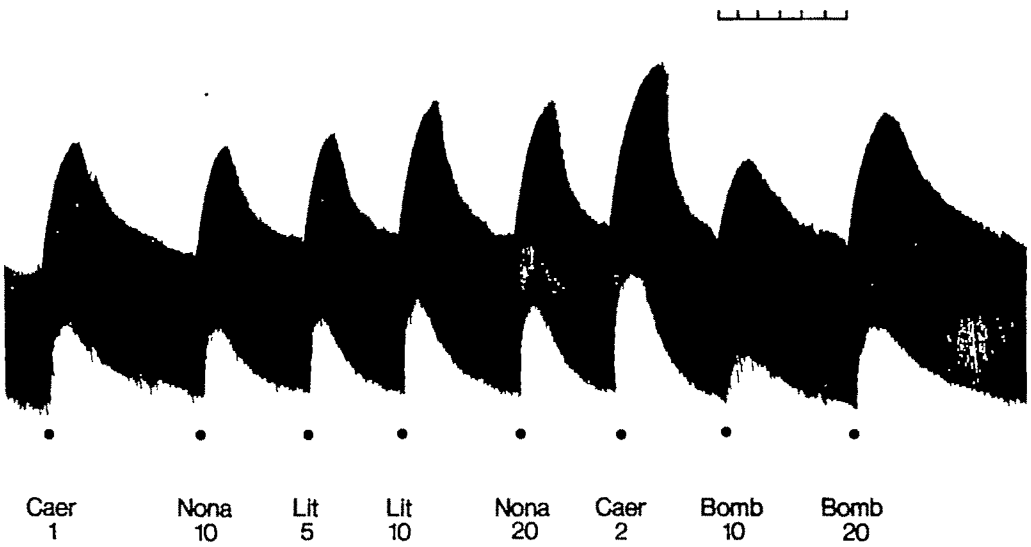

Figure 2 Guinea-pig anaesthetized with urethane. Responses of the gall bladder *in situ* to different intravenous doses (all in ng/kg) of bombesin (Bomb), litorin (Lit), bombesin nonapeptide (Nona) and caerulein (Caer). Time marks, 1 minute.

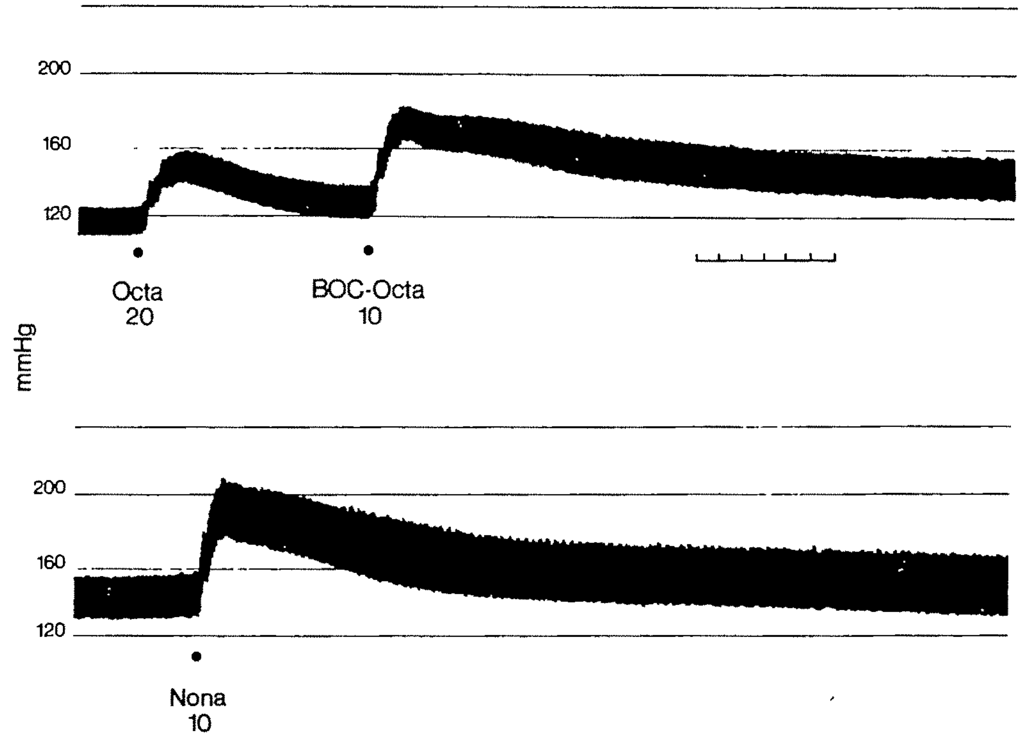

Figure 3 Blood pressure of a dog anaesthetized with sodium pentobarbitone (40 mg/kg, i.v.). Time marks, 1 minute. The effects of intravenous doses (in μg/kg) of octapeptide (Octa), BOC-octapeptide (BOC-Octa) and nonapeptide (Nona) of bombesin are shown. Note the increase in both intensity and duration of action produced by the passage from octapeptide to BOC-octapeptide and then to nonapeptide.

Dog Hexapeptide, BOC-hexapeptide, heptapeptide and BOC-heptapeptide were inactive up to 25 μg/kg given by rapid intravenous injection. Threshold doses for the octapeptide were 10 to 20 μg/kg. The relative potencies of the active peptides examined were as follows: bombesin = alytesin = nonapeptide > BOC-octapeptide > octapeptide (Figure 3). Equiactive doses of bombesin, nonapeptide and octapeptide produced effects of decreasing duration, in that order. Litorin was considerably less potent than bombesin, and its action less sustained.

Cat In two experiments the BOC-octapeptide showed 10-20% of the hypertensive activity of bombesin, and the nonapeptide 60%.

Rat The octapeptide possessed barely 3 to 5% of the hypertensive activity of bombesin, the nonapeptide 50%. Litorin was generally more potent than bombesin, but its action was less sustained.

Gastrin release and gastric acid secretion

The effects of the C-terminal nonapeptide of bombesin, infused at a rate of 10 ng kg^{-1} min^{-1} over a 60 min period were studied in gastric fistula dogs provided with Heidenhain pouches. Results are shown in Figure 4. On the whole, the nonapeptide produced the same release of gastrin as bombesin, at comparable infusion rates. However, gastric acid secretion was stimulated somewhat less by the nonapeptide, both in regard to peak response and to duration of effect.

Litorin was half as active as bombesin, and effects elicited by the peptide sharply decreased before the infusion of the peptide was stopped (Endean, Erspamer, Falconieri Erspamer, Improta, Melchiorri, Negri & Sopranzi, 1975).

Myo-electric activity of the dog duodenum

The C-terminal nonapeptide of bombesin showed approximately 75% of the activity of the parent peptide, and litorin 50% on the electric activity of the dog duodenum *in situ* (Caprilli *et al.*). The hepta- and the octapeptide were inactive at infusion rates of up to 50 ng kg^{-1} min^{-1}.

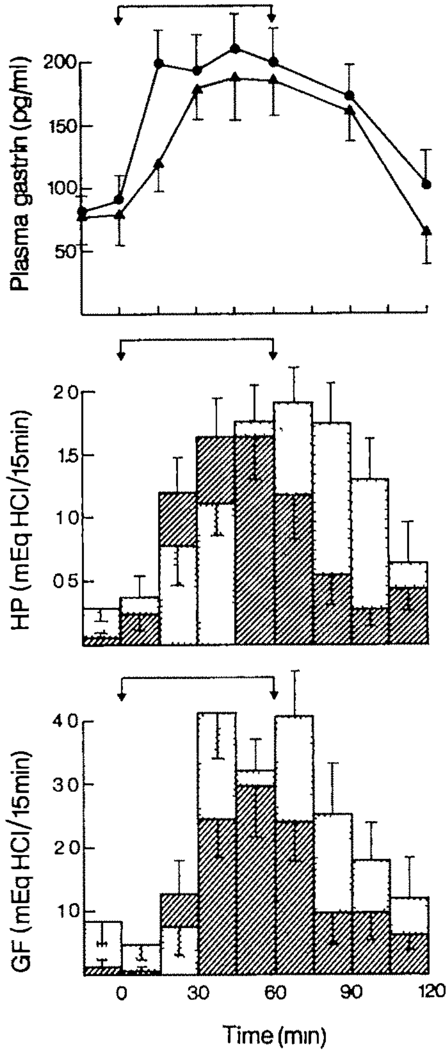

Figure 4 Gastric fistula dogs provided with Heidenhain pouches. Plasma gastrin levels and acid outputs, in the Heidenhain pouch (HP) and the main stomach (GF) following intravenous infusion, for 60 min, of 10 ng kg^{-1} min^{-1} of bombesin (▲, stippled areas) and bombesin nonapeptide (●, cross-hatched areas). Columns represent the mean of 2-3 measurements in each of 2-3 dogs. Vertical bars show s.e. mean.

Discussion

From the comparative bioassay of the 27 bombesin-like peptides examined in this study the following conclusions may be drawn:

(a) A minimum C-terminal sequence of seven amino acid residues was required for the appearance of bombesin-like effects. The C-terminal hexapeptide was quite inactive and similarly the N-terminal octapeptide (< 0.1%). The preparation most sensitive to the heptapeptide was the rat uterus.

(b) Addition of the glutamine residue to the N-terminus of the heptapeptide produced a 5- to 20-fold increase of activity on all preparations tested. Addition of an asparagine residue to the N-terminus of the octapeptide also produced a striking increase of activity on all preparations tested. The C-terminal nonapeptide of bombesin was as active as, or even more active, than bombesin on all isolated smooth muscle preparations examined. On *in vivo* test objects the nonapeptide had on a weight basis, 70 to 200% of the activity of bombesin.

(c) Protection of the N-terminal alanine residue of the hexapeptide with a *ter*-butyloxycarbonyl group (BOC-hexapeptide) did not cause the appearance of any activity. However, protection with BOC of the N-terminal tryptophan residue of the heptapeptide (BOC-heptapeptide) produced a 5- to 20-fold increase of activity, yielding a compound which was as active as the octapeptide itself; similarly the BOC-octapeptide was several times more potent than the octapeptide, approaching the activity of the nonapeptide. The BOC-nona-, deca- and endecapeptides, on the contrary, did not possess greater activity than the corresponding peptides lacking the BOC group.

Thus, the presence of the tryptophan residue seemed necessary for the appearance of the bombesin-like activity. Tryptophan could not be substituted by the BOC group. However, further lengthening of the peptide chain from the hepta- to the nona-peptide, which caused striking increase in activity up to an optimum in the nonapeptide, could be obtained not only by addition of amino acid residues, but also by addition of the BOC group.

(d) No appreciable differences in activity could be seen between the nona- and the decapeptide, and between the deca- and the endecapeptide. These peptides were also hardly distinguishable from each other from a qualitative point of view, for example in the appearance of tachyphylaxis and in the shape of the response.

(e) Replacement in the C-terminal octapeptide of bombesin of glutamine by asparagine or valine produced no change in activity, except perhaps in the stimulant action on the rat uterus, which appeared to be increased. Replacement of glutamine by phenylalanine, on the contrary, increased the activity on nearly all preparations tested.

It should be stressed that all the octapeptides examined differed from peptides having a longer chain of amino acid residues, in that their action was more rapid but less sustained, denoting either a more rapid inactivation or a less tenacious binding to the receptor sites.

(f) Addition of a 2,4-dinitrophenyl group (DNP) to the histidine residue of the BOC-nonapeptide drastically reduced the activity of the peptide, demonstrating the importance for bombesin-like activity of an intact histidine residue.

All conclusions listed under (c), (d), (e) and (f) were drawn from experiments on isolated smooth muscle preparations.

Parallel bioassay showed that the most simple bombesin-like peptide closely mimicking bombesin was the C-terminal nonapeptide of bombesin itself. Qualitatively its spectrum of activity was very similar to that of bombesin, except for the somewhat shorter duration of the effects *in vivo*; quantitatively its potency was, as already stated, equal to or greater than that of bombesin on isolated preparations, and more than half that of bombesin on gastrin release and electrical activity of the gut. Thus, it appears that the nonapeptide possesses all the prerequisites of an excellent substitute for bombesin in experimental as well as clinical investigation.

Two other natural bombesin-like peptides, in addition to bombesin, have been isolated by our group from the amphibian skin; the tetradecapeptide alytesin and the nonapaptide litorin, the smallest natural bombesin-like peptide so far found. Alytesin was indistinguishable from bombesin on all smooth muscle preparations tested. Litorin, differing from the C-terminal nonapeptide of bombesin in having the pyroglutamic acid^1 and phenylalanine^8 residues substituted for the asparagine^1 and leucine^8 residues, showed consistent differences from bombesin in its spectrum of biological activity. They have been described in detail in a preceding paper (Endean *et al.*, 1975).

This work was supported by grants from the Consiglio Nazionale delle Ricerche, Rome.

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Received April 10, 1975.
Revised May 27, 1975.)

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EFFECTS OF OESTRADIOL AND PROGESTERONE ON THE *in vitro* PRODUCTION OF PROSTAGLANDIN $F_{2\alpha}$ BY THE GUINEA-PIG UTERUS

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1 The effects of oestradiol and progesterone on the production of prostaglandin $F_{2\alpha}$ by the guinea-pig uterus *in vitro* have been studied.

2 Ovariectomized guinea-pigs were treated with oestradiol benzoate, progesterone, or a combination of these hormones. The uteri were homogenized and incubated and the prostaglandin $F_{2\alpha}$ produced was measured by radioimmunoassay or combined gas chromatography-mass spectrometry (g.c.-m.s.). Oestradiol treatment or progesterone followed by oestradiol caused a significant increase in prostaglandin $F_{2\alpha}$ synthesis compared with control values, whereas progesterone treatment caused no significant increase.

3 The uteri from guinea-pigs on day 7 of the oestrous cycle were split into four parts, and each part homogenized and incubated either alone or in the presence of oestradiol-17β, progesterone, or a combination of these hormones. Prostaglandin $F_{2\alpha}$ was measured by g.c.-m.s. Oestradiol was found to increase prostaglandin $F_{2\alpha}$ synthesis by the uterus whereas progesterone had no effect. However, progesterone did inhibit the response to oestradiol.

4 Oestradiol treatment of the guinea-pig uterus causes an increase in capacity to produce prostaglandin $F_{2\alpha}$ *in vitro*. The role of progesterone is difficult to interpret.

Introduction

Production of prostaglandin $F_{2\alpha}$ by the guinea-pig uterus *in vitro* increases at the end of the oestrous cycle (Poyser, 1972). Prostaglandin $F_{2\alpha}$ and oestradiol levels in utero-ovarian venous plasma also increase at this time, whereas progesterone levels fall (Blatchley, Donovan, Horton & Poyser, 1972; Joshi, Watson & Labhsetwar, 1973). These observations suggest that the synthesis and output of prostaglandin $F_{2\alpha}$ by the guinea-pig uterus may be controlled by the ovarian steroid hormones.

We, therefore, decided to measure the *in vitro* production of prostaglandin $F_{2\alpha}$ by the uteri of ovariectomized guinea-pigs after treatment with progesterone and oestradiol benzoate. We also measured the *in vitro* production of prostaglandin $F_{2\alpha}$ by the day 7 guinea-pig uterus with oestradiol and progesterone present in the incubate. These results have been reported briefly (Naylor, 1975).

Methods

The effect of in vivo *treatment with ovarian hormones*

Twenty virgin female guinea-pigs, which had normal oestrous cycles, were ovariectomized during the luteal phase of the cycle. The guinea-pigs were anaesthetized by injection of pentobarbitone sodium (30 mg/kg), and a dorsal incision was made on either side of the backbone. The fallopian tubes were ligatured in two places and severed between the ligatures, enabling the ovaries to be removed.

Four to eight weeks after surgery, the guinea-pigs were divided randomly into four groups and injected subcutaneously daily with 0.5 ml arachis oil containing steroids as follows: Group I—0.5 ml vehicle only for 10 days (control); Group II—0.5 ml vehicle for 7 days followed by oestradiol benzoate (10 μg daily for 3 days) in arachis oil; Group III—0.5 ml vehicle containing progesterone (2.5 mg daily) for 10 days; Group IV—0.5 ml vehicle containing progesterone (2.5 mg daily) for 7 days followed by 0.5 ml vehicle containing oestradiol benzoate (10 μg daily) plus progesterone (2.5 mg daily) for 3 days. Treatment for all groups thus lasted for 10 days.

On the 11th day after the start of treatment, all guinea-pigs were killed by stunning, then incising the neck. The uteri were removed, weighed, and homogenized in 15 ml Tyrode solution with a Fison's glass homogenizer. The homogenate was bubbled with O_2 and incubated at 37° C for 90 minutes. After incubation, each homogenate was adjusted to pH 4 with HCl and extracted immediately with solvent as described previously (Poyser, 1972).

After ethyl acetate extraction, most samples were assayed by radio-immunoassay (R.I.A.) (Blatchley & Poyser, 1974), though the antibody was solid-phased as described for ovarian steroid hormones by Bolton, Dighe & Hunter (1975). Some samples, however, were assayed by combined gas chromatography-mass spectrometry (g.c.-m.s.) with a Finnegan 3000D mass spectrometer. To these samples was added 1 μg deuterated (d_4) prostaglandin $F_{2\alpha}$ before extraction. After extraction the prostaglandins were prepared as the methyl ester-trimethylsilyl ether (Me-TMS), (Thompson, Los & Horton, 1970; Blatchley, *et al.*, 1972) and assayed by single ion monitoring of the peaks at 423 (M-(90 + 71) for the non-deuterated (d_0) prostaglandin $F_{2\alpha}$ and 427 (d_4-prostaglandin $F_{2\alpha}$) as described by Hensby & Naylor, (1974).

Comparable results were obtained by R.I.A. and g.c.-m.s.

The effect of in vitro *treatment with ovarian hormones*

Six virgin female guinea-pigs which had normal oestrous cycles were killed on day 7 of the oestrous cycle (oestrus = day 1) by stunning, then incising the neck. The uteri were removed and each uterus was divided into four pieces. The portions from each uterus were weighed, then homogenized separately in 15 ml Krebs solution, to which had been added 0.3 ml ethanol containing steroids as follows: Group I–0.3 ml ethanol only (control); Group II–oestradiol-17β to give a final concentration of 100 μg/ml incubate; Group III–progesterone to give a final concentration of 500 μg/ml of incubate; Group IV–oestradiol-17β (100 μg/ml) and progesterone (500 μg/ml). The procedure was designed so that one portion of each uterus was included in each group.

After homogenization, the homogenates were bubbled with 95% O_2 and 5% CO_2 and incubated at 37°C for 90 minutes. Following solvent extraction, the samples were further purified by silicic acid column chromatography (Poyser, 1972), and the prostaglandin fractions assayed by g.c.-m.s. as in the previous experiment.

All results were analysed by Student's t test. Oestradiol and progesterone were purchased from Koch-Light Laboratories Ltd., and oestradiol benzoate from Sigma Chemical Company.

Results

The effect of in vivo *treatment with ovarian hormones*

The results are shown in Table 1. Treatment with the two hormones caused the uteri to increase in weight compared with control values, the greatest increase being produced after treatment with oestradiol benzoate alone. Progesterone treatment caused a smaller increase in uterine weight and, in addition, was found to inhibit partially the increase in uterine weight in response to oestrogen treatment. These are well known effects of the ovarian steroid hormones.

Due to this variation in uterine size after treatment with the two hormones, the amounts of prostaglandin $F_{2\alpha}$ produced by the uteri have been expressed both as ng/uterus and as ng/100 mg tissue. Total prostaglandin $F_{2\alpha}$ production/uterus was greatest in animals treated with either oestradiol benzoate, or progesterone followed by oestrogen. There was no significant difference between these two treatments although both produced significantly more prostaglandin $F_{2\alpha}$ ($P < 0.01$) than animals treated with only arachis

Table 1. The *in vitro* production of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) by the uterus of the ovariectomized guinea-pig after treatment with oestradiol and progesterone.

Treatment (No. of animals)	*Average weight of uteri (g) ± s.e. mean*	*Total $PGF_{2\alpha}$ production (ng/uterus) ± s.e. mean*	*ng $PGF_{2\alpha}$/100 mg tissue ± s.e. mean*
Control (4)	0.26 ± 0.02	250.8 ± 23.4^a	97.6 ± 8.7^d
Oestradiol benzoate (6)	1.00 ± 0 08	2009.3 ± 212.9^b	205.8 ± 27.1^e
Progesterone (6)	0.58 ± 0.04	641.7 ± 90 7^c	109.0 ± 12.6^d
Progesterone plus oestradiol benzoate (4)	0.71 ± 0.05	1478.3 ± 142.0^b	208.3 ± 8.6^e

Values in the same vertical column with the same superscript are not significantly different.

oil. Progesterone treatment alone also caused a significant ($P < 0.01$) increase over control values, but this production was significantly lower than after oestrogen or progesterone plus oestrogen treatment ($P < 0.01$).

When expressed in terms of ng prostaglandin F$_{2\alpha}$ production/100 mg tissue, the results clearly show that treatment with oestradiol benzoate, or progesterone followed by oestradiol benzoate caused a significant ($P < 0.05$) twofold increase in prostaglandin F$_{2\alpha}$ synthesis compared with uteri from animals treated with either arachis oil or progesterone. There was no significant difference between production levels after oestrogen, or progesterone plus oestrogen treatment. Similarly there was no significant difference between control levels, and levels after progesterone treatment.

The effect of in vitro *treatment with ovarian hormones*

The results are shown in Table 2. The control values are in good agreement with values obtained previously (Poyser, 1972; Hensby & Naylor, 1974). Oestradiol treatment causes a significant ($P < 0.01$) increase in production of prostaglandin F$_{2\alpha}$ by the day 7 guinea-pig uterus compared with control values. Treatment with progesterone alone did not cause any significant change in production of prostaglandin F$_{2\alpha}$, and values were significantly lower ($P < 0.01$) than after oestrogen treatment alone. However, progesterone abolished the increase in prostaglandin F$_{2\alpha}$ production caused by oestrogen, as the results of the combined treatment showed. Production of prostaglandin F$_{2\alpha}$ after this combined treatment was significantly lower ($P < 0.05$) than control values. It was also lower than prostaglandin F$_{2\alpha}$ production after progesterone treatment alone, though this difference was not significant.

Table 2. The *in vitro* production of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) by the day 7 guinea-pig uterus in the presence of oestradiol and progesterone.

Treatment (No. of replicates = 6)	*ng PGF$_{2\alpha}$/100 mg tissue ± s.e. mean*
Control	52.1 ± 3.4^{f}
Oestradiol	90.1 ± 5.2^{g}
Progesterone	$55.0 \pm 7.9^{f,h}$
Oestradiol plus progesterone	33.7 ± 5.8^{h}

Values with the same superscript are not significantly different.

Discussion

In the guinea-pig, the increase in oestradiol secretion from the ovary towards the end of the oestrous cycle (Joshi *et al.*, 1973) may be the stimulus for increased prostaglandin F$_{2\alpha}$ production and release from the uterus at this time (Poyser, 1972; Blatchley *et al.*, 1972). Bland & Donovan (1968, 1970) have shown that injections of oestradiol benzoate on days 3-11 of the oestrous cycle, or a single injection on day 3 led to regression of the corpora lutea of guinea-pigs, an effect abolished by hysterectomy. Oestradiol benzoate injections early in the cycle also gave rise to increased prostaglandin F$_{2\alpha}$ levels in utero-ovarian venous blood (Blatchley, Donovan, Poyser, Horton, Thompson & Los, 1971; Blatchley *et al.*, 1972) though not in hysterectomized animals, indicating that the prostaglandin F$_{2\alpha}$ comes from the uterus.

Treatment of guinea-pigs with progesterone from the day of oestrus resulted in a shortening of the oestrous cycle (Woody, First & Pope, 1967; Ginther, 1969a), but other workers have shown that progesterone given immediately after this period has no effect on the life-span of the corpus luteum in intact animals (Bland & Donovan, 1968, 1970; Choudary & Greenwald, 1968; Ginther, 1969b). Similarly, 5 mg implants of progesterone in intact guinea-pigs had no effect on luteolysis, although ovulation was completely inhibited (Deanesly, 1968).

The present studies involving the treatment of ovariectomized guinea-pigs with oestrogen and progesterone indicate that the capacity of the uterus to synthesize different amounts of prostaglandin F$_{2\alpha}$ per unit weight of tissue, *in vitro*, is controlled by oestrogen, and progesterone is not a prerequisite for increased synthesis. However, progesterone does appear to be required for the synthesis and release of prostaglandin F$_{2\alpha}$ *in vivo* as shown by Blatchley & Poyser (1974). It is possible, therefore, that the rate-limiting step in the release of prostaglandin F$_{2\alpha}$ from the uterus of the guinea-pig is the availability of arachidonic acid, which consequently may be governed by progesterone.

It cannot be overlooked that decreased metabolism of prostaglandin F$_{2\alpha}$ by the uterus could account for the results obtained. However, metabolism of prostaglandin F$_{2\alpha}$ by the guinea-pig uterus is only 5 to 10% (F.M. Maule Walker & N.L. Poyser, unpublished observations). Therefore it would appear that complete inhibition of metabolism could not account for the increase in observed levels of prostaglandin F$_{2\alpha}$ and the results are better interpreted as due to an increase in synthesis of prostaglandin F$_{2\alpha}$.

A study by Barcikowski, Carlson, Wilson & McCracken, (1974) showed that prostaglandin $F_{2\alpha}$ output by the sheep uterus increased between 60 and 90 min after the start of infusion of oestradiol. Progesterone levels were high at that time. Our present studies have shown that a large dose of oestradiol *in vitro* also caused an increase in the capacity of the guinea-pig uterus to synthesize prostaglandin $F_{2\alpha}$ after a similar time interval. The effect of oestradiol was surprisingly abolished by the presence of progesterone in the incubate. Similar inhibition by progesterone of prostaglandin F synthesis has been demonstrated by Cane & Villee (1975) using human endometrial slices cultured *in vitro.* At present it is difficult to interpret this inhibitory action of progesterone.

The authors gratefully acknowledge the encouragement of Professor E.W. Horton, whose grant from the Medical Research Council made this work possible. Authentic prostaglandins were kindly supplied by Dr J.E. Pike of the Upjohn Company, Kalamazoo, Michigan, U.S.A. B.N. is an M.R.C. research student and N.L.P. is an M.R.C. research fellow.

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(Received June 11, 1975.
Revised July 17, 1975)

Br. J. Pharmac. (1975), 55, 233-238

RAPID REDUCTION OF PROSTAGLANDIN 15-HYDROXY DEHYDROGENASE ACTIVITY IN RAT TISSUES AFTER TREATMENT WITH PROTEIN SYNTHESIS INHIBITORS

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1 The capacity of rat kidneys to metabolize prostaglandins is rapidly lost when *de novo* protein synthesis is reduced by cycloheximide and puromycin. Enzyme activity declines to about half control values 45-75 min after a single dose of cycloheximide. Prostaglandin metabolism by the lung is also diminished.

2 These inhibitors apparently act by preventing the synthesis of new prostaglandin 15-hydroxy dehydrogenase enzyme.

3 The RNA synthesis inhibitor actinomycin D has no effect on metabolism.

4 It is concluded that prostaglandin dehydrogenase is a short-lived enzyme in the cell whose replacement depends upon *de novo* protein (but not RNA) synthesis. The implications of this finding are discussed.

Introduction

The initial step in the metabolic degradation of prostaglandins is oxidation of the C-15 alcohol (Änggård & Samuelsson, 1964, 1966; Hamberg & Samuelsson, 1971a). This reaction is catalyzed by prostaglandin 15-hydroxy-dehydrogenase (PGDH), an enzyme found in high concentrations in the lung and kidney (Änggård, Larsson & Samuelsson, 1971). This oxidation reaction can result in a substantial loss of biological activity (Änggård, 1966) and plays an important part in the inactivation of prostaglandins in man and animals (Green & Samuelsson, 1971; Hamberg & Samuelsson, 1971b, 1972).

Proteins are synthesized by all viable cells, and a state of dynamic equilibrium exists such that the continual degradation which occurs is balanced by *de novo* synthesis. The 'lifespan' of an enzyme, as determined by the rapidity of its synthesis and destruction, may be an important regulatory mechanism. One way of estimating this lifespan is to inhibit *de novo* protein or RNA synthesis using specific blocking agents, such as cycloheximide, puromycin or actinomycin D (see Reich, 1963; Vazquez, 1974).

Whilst investigating the effect of protein synthesis inhibitors on the ability of rat kidney homogenates to synthesize prostaglandins, we observed that the rate of prostaglandin metabolism apparently fell. We now present evidence that PGDH is a short-lived enzyme and that its continued activity depends on continual protein, but not RNA synthesis within the cell.

A preliminary account of this work has been presented to the Society (Blackwell, Flower, Parsons & Vane, 1975).

Methods

Measurement of prostaglandin metabolism

Rats (male, Wistar strain, weighing between 150-250 g) were killed by a blow on the head. The kidneys (or in one experiment, the lungs) were removed, immediately placed on ice and homogenized within 5 min in 4 vol of ice-cold 100 mM pH 7.5 Tris buffer. The resultant suspensions were then cetrifuged at 1,000 g_{av} to remove coarse cellular debris and the supernatant re-centrifuged for 1 h at 100,000 g_{av} in a Beckman Spinco ultra-centrifuge. The resultant pellet was discarded, and the supernatant, which contained PGDH, was used for assays of prostaglandin metabolism. The supernatant was stored on ice until required for use since enzyme activity was very labile; when kept at room temperature, about 50% activity was lost within 30 min of centrifugation. Reaction sets for the radioisotope assay comprised the following components: supernatant, 1 ml; NAD$^+$, 280 pmol; [^{3}H]-prostaglandin E$_2$, 0.1 μCi; unlabelled prostaglandin E$_2$, 28 pmol. After the contents were thoroughly mixed the tubes containing the reaction mixture were incubated in a shaking waterbath at 37° C for varying time periods. The reaction was terminated by acidifying the mixture to pH 3 with 1 N HCl and the unreacted prostaglandin E$_2$ and its metabolites were extracted by mixing for 30 s with 1.5 ml of ethyl acetate. This procedure reproducibly extracts more than 95% of labelled products. After mixing, the phases were separated by centrifugation, 1 ml of the organic layer was removed and mixed with 5 μg each of unlabelled prostaglandin E$_2$ and 15-keto prostaglandin E$_2$. The organic solvents were removed in a vacuum dessicator and the dried residue redissolved in 50 μl of ethanol and spotted quantitatively onto a plastic-backed silica gel thin layer chromatography (t.l.c.) plate. After development in iso-octane, ethyl acetate, acetic acid, and water (5:11:2 10), the plates were dried and zones corresponding to authentic prostaglandin markers were visualized by brief exposure to iodine vapour. Those zones corresponding to prostaglandin E$_2$ and its 15-keto metabolite were cut out with scissors and after spontaneous evaporation of the iodine, the radioactivity in each zone was estimated by liquid scintillation counting using Beckman cocktail 'D' and a Beckman LS-150 scintillation counter; ct/min were converted to d/min using a quench correction curve and external standards.

In one experiment prostaglandin metabolism was quantitated biologically. Reaction sets in this instance comprised 2.8 nmol prostaglandin E$_2$ and 28 nmol NAD$^+$. After solvent extraction the prostaglandins were bioassayed on the rat fundic strip, treated with a mixture of antagonists to make the assay more specific (see Gilmore, Vane & Wyllie, 1966).

Estimation of protein synthesis

Rat kidneys were finely chopped with a scalpel blade and incubated in test tubes containing 1 ml of 100 mM pH 7.5 Tris buffer and 0.1 μCi of [^{14}C]-lysine. After 1 h, the slices were homogenized in the buffer using a glass homogenizer, and the proteins precipitated with 5 volumes of ice-cold 1 M perchloric acid. The precipitate was sedimented in a bench centrifuge, and the supernatant discarded. The precipitate was washed twice with 5 volumes of 0.5 M perchloric acid and 5 volumes ethanol/ether (1/1), and the final pellet solubilized with 'NCS' or concentrated sodium hydroxide. The radioactivity in aliquots of the digest was estimated by liquid scintillation counting after neutralization with acetic acid, and the protein content was estimated by the biuret reaction (Gornall, Bardawill & David, 1949).

Estimation of RNA synthesis

The procedure was identical to that outlined in the previous section except that the incorporation of 0.1 μCi of [^{3}H]-uridine was measured. Radioactivity in the acid insoluble material was again estimated by liquid scintillation counting techniques

Drugs

The following compounds were used: cycloheximide, puromycin dihydrochloride, actinomycin D and β-NAD$^+$, (Sigma); [5,6,8,11,12,14, 15-^{3}H]-prostaglandin E$_2$ (batch 4, 160 Ci/mmol); [5-^{3}H]-uridine (batch 93, 29 Ci/mmol), [U-^{14}C]-(L)-lysine (batch 52, 348 mCi/mmol), (Radiochemical Centre, Amersham); plastic-backed silica gel (type 6060) t.l.c. plates (Eastman Kodak); unlabelled prostaglandin E$_2$ and F$_{2\alpha}$ (Cambrian Chemicals); and NCS (Amersham-Searle). The 15-keto derivative of prostaglandin E$_2$ was prepared by oxidation of the parent compound in chloroform by manganese dioxide All other reagents were of 'Analar' grade, or the highest purity obtainable.

Results

Time course for inhibition of protein and RNA synthesis

In an initial series of experiments the time course for inhibition of protein (see Figure 1) and RNA synthesis was established. A single dose of cycloheximide (20 mg/kg i.p.) was sufficient to produce a progressive fall in protein synthesis which reached about 50% of control values 1-2 h after injection and fell to about 20% after 4 hours. Most rats were dead within 10 h and even at 2-4 h exhibited lassitude and diarrhoea. Puromycin was a much less effective inhibitor but hourly doses of 50 mg/kg (i.p.) produced a similar effect to that of cycloheximide, and the incidence of observable side-effects was reduced. The inhibition of protein synthesis by both drugs using the dose schedule above was quite reproducible but was checked for each experiment (see Tables1-3). PGDH activity was usually measured 2 h after treatment (i.e. when protein synthesis was less than 50% of control values).

The inhibitory effect of actinomycin D on RNA synthesis had a much longer latency. It was

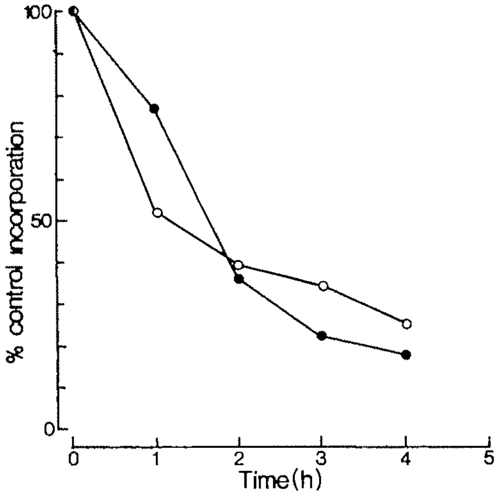

Figure 1 Time course of the effect of protein synthesis inhibitors puromycin (○) and cycloheximide (●) on *de novo* protein synthesis by rat kidney. For dosage see results section. The results are expressed as % control incorporation of [^{14}C]-lysine/mg protein Each point is the mean of 2-4 determinations.

necessary to dose rats (2 5 mg/kg i.p.) 12 h prior to the experiment in order to achieve a greater than 50% reduction of [^{3}H]-uridine incorporation. The effect of actinomycin D on protein synthesis at this time was very small (less than 5%) or undetectable. A high mortality rate was observed in rats treated for longer than 12 h with actinomycin D.

In these initial experiments the kidney protein content of control rats was compared with those receiving protein or RNA synthesis inhibitors but no differences were detected.

Kinetics of [^{3}H]-prostaglandin E_2 oxidation

Under the reaction conditions described in the methods section the oxidation of [^{3}H]-prostaglandin E_2 was linear for 4 to 5 min during which one third to one half of the labelled substrate was consumed; thereafter the rate of oxidation declined (Figure 2). Reaction mixtures were, therefore, routinely incubated for 4 minutes. At this time the labelled substrate was transformed into a single less polar product (as judged by t.l.c.) which had an R_F value identical to that of authentic 15-keto prostaglandin E_2. The formation of this product was NAD$^+$-dependent. The reduction products obtained after treatment of the isolated metabolite with sodium borohydride (principally $F_{2\alpha}$-β) had chromatographic mobilities identical to those products obtained after reduction of the authentic compound. It is virtually certain, therefore, that this product was the 15-keto metabolite of prostaglandin E_2.

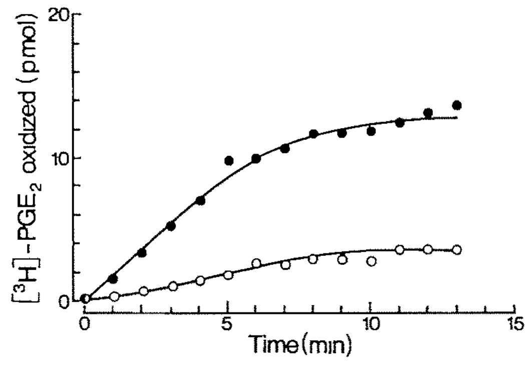

Figure 2 Oxidation of prostaglandin E_2 by high-speed supernatant of rat kidney homogenates of control rats (●) and of rats treated with cycloheximide 2 h before the experiment (○). Incubation conditions are described in the methods section. Each point is the mean of duplicate determinations.

Effect of cycloheximide on [^{3}H]-prostaglandin E_2 oxidation

Figure 2 shows the rates of [^{3}H]-prostaglandin E_2 oxidation in the high-speed supernatant of kidneys from control rats, and kidneys from rats pretreated with cycloheximide. There was a dramatic reduction in oxidation rate from 1.75 pmol [^{3}H]-prostaglandin E_2/min for control rats, to 0.31 pmol [^{3}H]-prostaglandin E_2/min for rats treated with cycloheximide.

In two sets of experiments the loss of oxidizing activity with time was calculated. Seven rats were injected with cycloheximide and killed at 20 min intervals. The kidneys were removed, processed and the enzyme activity measured. The ability to oxidize [^{3}H]-prostaglandin E_2 was reduced to half of the control levels 45-75 min after a single dose of the drug.

Direct action of cycloheximide on PGDH

The reduction in PGDH activity by cycloheximide could have been due to a direct antagonism of the drug or a metabolite on the enzyme rather than an effect on protein synthesis. To exclude the former possibility, control experiments were performed with high-speed supernatants of kidney homogenates from untreated rats with the addition of cycloheximide directly to the reaction mixture. In concentrations as high as 2.5 mg/ml cycloheximide caused less than 5% inhibition of PGDH activity. Thus, the effects of cycloheximide *in vivo* (at a maximum dose of 20 mg/kg) were not due to a direct inhibition of the enzyme.

Effect of puromycin on PGDH activity

To exclude the possibility that the effect of cycloheximide was due to formation of a metabolite, puromycin, a protein synthesis inhibitor of a different chemical structure and mode of action (Vazquez, 1974) was tested. Table 1 shows that this drug had qualitatively the same effect as cycloheximide; there was a reduction in protein synthesis (69% inhibition) as well as in oxidizing capacity (56% inhibition).

Effect of cycloheximide on PGDH in the lung

To discover whether the action of cycloheximide was restricted to the kidney we performed one experiment in which the PGDH activity in rat lung was measured after cycloheximide. Table 2 shows that when lung protein synthesis was inhibited by 64%, PGDH activity was reduced by 67%.

Effect of actinomycin D on kidney PGDH activity

The effect of actinomycin D, an inhibitor of RNA (but not protein) synthesis, is shown in Table 3. Using the dose schedule described above, RNA synthesis (as quantitated by [^{3}H]-uridine incorporation) was inhibited by 53%. Protein synthesis, however, fell by only 4% and there was no reduction in PGDH activity.

Controls for isotope dilution artifacts

An inherent flaw in all isotope assays using crude enzyme preparations is that the extent to which the isotope is metabolized may be influenced by the pre-existing pool size of unlabelled material in the enzyme preparation. In the present experiments for example, the amount of [^{3}H]-prostaglandin E_2 metabolism could be influenced by the amount of unlabelled prostaglandin E_2 (or other prostaglandins) present

Table 1. Effect of puromycin on rat kidney protein synthesis and prostaglandin 15-hydroxy dehydrogenase (PGDH) activity

	[^{3}H]-PGE$_2$ oxidized (pmol) (mean ± s.e. mean)	*[^{14}C]-lysine incorporated (pmol)*
Control	14.08 (± 0.12) (*n* = 4)	33.71 (*n* = 2)
Plus puromycin	6.18 (± 0 14) (*n* = 5)	10.23 (*n* = 2)

Table 2. Effect of cycloheximide on rat lung protein synthesis and prostaglandin 15-hydroxy dehydrogenase (PGDH) activity

	[^{3}H]-PGE$_2$ oxidized (pmol)	*[^{14}C]-lysine incorporated (pmol)*
Control	8.79 (*n* = 2)	42.40 (*n* = 2)
Plus cycloheximide	3.26 (*n* = 2)	15.39 (*n* = 2)

Table 3. Effect of actinomycin D on rat kidney protein and RNA synthesis and prostaglandin 15-hydroxy dehydrogenase (PGDH) activity

	[^{3}H]-PGE$_2$ oxidized (pmol) (mean ± s.e. mean)	*[^{3}H]-uridine incorporated (fmol)*	*[^{14}C]-lysine incorporated (pmol)*
Control	15.96 (± 0.19) (*n* = 5)	224.29 (*n* = 2)	30.44 (*n* = 2)
Plus actinomycin	16.12 (± 0.18) (*n* = 5)	104.73 (*n* = 2)	28.79 (*n* = 2)

in the high-speed kidney supernatant. Rat kidneys are rich in prostaglandin synthetase and it could be argued that if cycloheximide were stimulating renal prostaglandin synthesis the amount of [^{3}H]-prostaglandin E_2 metabolized might be reduced thus giving apparently similar results to the findings reported here.

We have therefore checked the results of the cycloheximide experiments using bioassay techniques to measure the total prostaglandin content of the reaction mixture. Similar results were obtained to those obtained by the isotope method; the metabolism in the control samples was 457.1 pmol ($n = 3$) and in samples from cycloheximide-treated rats it was 174.4 pmol ($n = 4$).

Discussion

The addition of [^{3}H]-prostaglandin E_2 to high-speed supernatants of rat kidney resulted in rapid metabolism to the 15-keto derivative. This metabolism was greatly depressed when rats were pretreated with the protein synthesis inhibitor, cycloheximide. This effect was not due to an isotope dilution artifact nor to a direct inhibition of the enzyme by cycloheximide. The fact that puromycin was also effective despite its different chemical structure and mode of action (Vazquez, 1974) makes it unlikely that the effect was due to formation of an inhibitory metabolite. The fact that we have obtained the same effect on kidney slices *in vitro* (Blackwell *et al.*, 1975), makes it even more unlikely that cycloheximide is metabolized to an active inhibitor of PGDH. When measured under constant conditions a reaction rate is proportional to the amount of enzyme present, and we suggest, therefore, that the results are due to a decreased synthesis of new PGDH enzyme. This effect is probably a general one, for lung as well as kidney PGDH activity was depressed by pretreatment with cycloheximide. The inability of actinomycin D to affect PGDH activity (despite its powerful inhibitory action on RNA synthesis) suggests that the RNA template for PGDH synthesis is relatively stable.

The time course studies indicate that PGDH is a very short-lived enzyme with a half-life of approximately 1 h after cycloheximide injection. The life of proteins within cells may vary from less than 1 h to 20 days or more (Schapira, Kruh, Dreyfus & Schapira, 1960; Kenney, 1962; Nicolette & Mueller, 1966; Gorski & Morgan, 1967). The short life span of PGDH may be due to an inherent instability of the protein molecule. Certainly, the enzyme deteriorates rapidly after purification and even preparations stored at 4°C have a life of only 60 to 80 days (Marazzi & Andersen, 1974). Our own observations support the concept that PGDH is unstable, for when the crude enzyme was kept at room temperature (about 21°C) it rapidly lost its capacity to oxidize [^{3}H]-prostaglandin E_2. Metabolism fell to about half the control values in 30 min and was virtually absent after 1 hour. The enzyme is thought to contain sulphydryl groups (Marazzi & Andersen, 1974) and there is a possibility, therefore, that the loss of activity occurs as a result of oxidative damage. Recently, Parkes & Eling (1975) have shown that exposure of guinea-pigs to 100% oxygen for 48 h results in a considerable loss of lung PGDH activity. Another possibility is that PGDH is degraded by cytoplasmic proteolytic enzymes for Katunuma, Kito & Kominami (1971) have described a proteolytic enzyme which specifically inactivates NAD^+-dependent dehydrogenases.

What are the physiological implications of this work? PDGH belongs to that group of enzymes (which includes phosphodiesterase, acetylcholinesterase, monoamine-oxidase and kininase) responsible for the inactivation of endogenous substances of high biological activity (Marazzi & Andersen, 1974). Thus the mechanism by which its catalytic activity is controlled is clearly of interest. One possibility is that the synthesis of PGDH is controlled by blood concentrations of steroid or other hormones (Bedwani & Marley, 1975).

Another implication is that any agent which decreases cellular protein synthesis would decrease PGDH activity and thus result in elevated prostaglandin levels. Many antibiotics as well as some other compounds such as diphtheria toxin block protein synthesis (Vazquez, 1974). In this connection it is interesting to note that Nakano & Prancan (1973) have demonstrated that PGDH activity in the lung and kidney was impaired in rats after endotoxin administration.

A further implication of the work described here is that irreversible inhibitors of PGDH are likely to have short duration of action.

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(Received May 9, 1975.
Revised June 20, 1975.)

PROCEEDINGS OF THE
British Pharmacological Society

15th-17th September, 1975

THE UNIVERSITY OF CAMBRIDGE

COMMUNICATIONS

In communications with more than one author, an asterisk (*) denotes the one intending to present the work.

Prostaglandin D_1 inhibits the increase in vascular permeability in rat skin produced by prostaglandin E_1, E_2 and D_2

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Increased vascular permeability is induced by intradermal injection of prostaglandin (PG) E_1, E_2 (Crunkhorn & Willis, 1971a) or D_2 (Kingston, Flower & Harvey, 1975). $PGF_{2\alpha}$ diminishes the cutaneous vascular effects of PGE_1, PGE_2 (Crunkhorn & Willis, 1971b) histamine or bradykinin (Thomas & West, 1974). PGD_1 also reduces the cutaneous vascular effects of prostaglandins E_1, E_2 and D_2 in rat skin. We have compared the effects of PGD_1 and $F_{2\alpha}$ on increased vascular permeability, as measured by extravasation of ^{125}I-albumin.

Table 1 Extravasation of ^{125}I-albumin at the sites of intradermal injection (n = 10)

	*Counts in lesion** / *Counts in 10 µl blood*		
	Alone	*+ 0.5 µg PGD_1*	*+ 0.5 µg $PGF_{2\alpha}$*
100 ng PGE_1	9.0	2.7	2.9
1 µg PGE_2	28.8	10.7	8.2
1 µg PGD_2	10.7	3.8	3.0
1 µg Bradykinin	6.1	5.9	4.6
10 µg Histamine	3.6	3.7	1.3
0.5 µg PGD_1	0.4		
0.5 µg $PGF_{2\alpha}$	0.3		
Saline	0.3		

* Values corrected for a background of 1.6.

Olac rats (approximately 200 g weight) were lightly anaesthetized with ether and injected intravenously with ^{125}I albumin (5 μCi) and Evans blue (40 mg/kg). Drugs were then injected in 0.1 ml saline intradermally into the shaved abdominal skin. Twenty minutes later, the animals were killed and the skin removed. Discs of skin were excised which included the whole of the lesion (as indicated by blueing). The excised skin was wrapped in a single layer of parafilm and the radioactivity measured in a gamma counter. The radioactivity in 10 μl of blood was measured as a reference.

Intradermal injection of PG E_1, E_2 and D_2 produced increases in vascular permeability. These effects were reduced by admixture with PGD_1 (Table 1).

PGD_1 did not modify the increased vascular permeability induced by histamine or bradykinin, as would be expected if it was acting through vasoconstriction. $PGF_{2\alpha}$ reduced the effects of histamine and bradykinin as well as prostaglandins E_1, E_2 and D_2 showing that its effect was less specific than that of PGD_1. These results suggest a role for PGD_1 and $PGF_{2\alpha}$ as modulators of skin inflammation.

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A comparison of the histopathological effects of anaphylatoxin (AT) and prostaglandins E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) in guinea-pig lungs

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In a previous study it was shown that AT induced the release of a substance with prostaglandin-like activity in isolated perfused guinea-pig lungs (Sackeyfio, 1972). Other investigations have also shown that the complex effects of AT in the guinea-pig lead not only to the release of the bronchoconstrictor substance, histamine (Sackeyfio, 1971) but also to the stimulation of adrenergic mechanisms which are predominantly bronchodilator (Hicks & Sackeyfio, 1971). However, the dominant symptomatic feature of (AT) administration in the guinea-pig is intense respiratory distress. The present investigation was undertaken with the view that a comparison of the histopathological effects of AT with those of known or suspected mediators of AT activity might provide further elucidation of the mechanisms involved in AT activity.

AT was prepared as previously reported (Sackeyfio, 1971). Groups of guinea-pigs were anaesthetized with pentobarbitone (60 mg/kg i.p.) and injected i.v. with one or other of the following: AT (0.5 ml/kg); rat native serum (0.5 ml/kg), histamine (20 μg/kg), PGE_2 (1 mg/kg) or $PGF_{2\alpha}$ (1 mg/kg). The lungs were isolated, fixed in 10% buffered formalin for 24 h, embedded in wax, sections were stained with haematoxylin and eosin and examined microscopically. Lungs from untreated guinea-pigs were similarly prepared for comparison.

Examination of the slides showed that AT, histamine, PGE_2 and $PGF_{2\alpha}$ induced varying degrees of bronchoconstriction and thickening of the bronchial muscle (the order of intensity being AT $>$ histamine $= PGF_{2\alpha} > PGE_2$). Intense peribronchial oedema and vasoconstriction were observed only in the AT- and $PGF_{2\alpha}$-treated animals, but not in the other groups of guinea-pigs. AT, histamine and $PGF_{2\alpha}$ induced perivascular oedema, thickening of the vascular wall and intense emphysema but PGE_2 had no such effects. Neither the untreated nor the native serum treated animals showed any of these histopathological effects.

The results of this study showed that the histopathological effects of AT were mimicked entirely by $PGF_{2\alpha}$, partially by histamine but hardly at all, by PGE_2.

Mepyramine (0.5-4 mg/kg) abolished the effects of histamine but only partially reduced the AT-induced effects. The AT-induced oedema was particularly resistant to the inhibitory action of mepyramine. On the other hand the anti-inflammatory agents indomethacin and phenylbutazone (0.1-4 mg/kg) abolished the responses to the prostaglandins and effectively inhibited the AT-induced oedema.

It may be concluded from these observations that the histopathological effects of AT are probably mediated through the release of histamine and $PGF_{2\alpha}$, the latter mediator possibly contributing more to the perivascular and peribronchial oedema.

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Release of prostaglandins in the knee joint of the dog during local Shwartzman-like reaction

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The Shwartzmann phenomenon, originally described in the rabbit (Shwartzman, 1928), is a two-stage reaction· the first event consists of a local inflammatory reaction in the skin after an intradermal injection of bacterial endotoxin; the second one consists of a haemorrhagic necrosis of the prepared skin area following an intravenous injection of the endotoxin, 18-24 h after the intradermal injection. Since endotoxin produces an inflammation in the knee joint of the dog (Morgan & Bennett, 1947; Van Arman, Carlson, Kling, Allen & Bondi, 1974) during which prostaglandins (PG) are locally released (Herman & Moncada, 1975), we decided to study the possibility of producing a Shwartzman-like reaction in the dog's knee joint, the release of PG's during its development and the effect of local treatment with indomethacin, which has been shown to inhibit the Shwartzman reaction in rabbit skin (Van Arman, Carlson, Brown & Itkin, 1970).

Mongrel dogs of either sex were anaesthetized with thiopentone (10 mg/kg intravenously). A 'preparatory' injection of endotoxin (E. coli O 111 B4, Difco; 2.5 ng/kg, 9 experiments; 25 ng/kg, 12 experiments) in 0.5 ml.of sterile saline was given in one of the knee joint cavities. Symptoms of incapacitation ranging from limping, occasional 3-legged gait to complete 3-legged gait were observed in all animals as described previously (Herman & Moncada, 1975). All animals received an intravenous 'provocative' injection of 250 ng/kg endotoxin 18-24 h later. In 15 out of 21 animals incapacitation occurred which started about 1 h after the injection and lasted for about 3-4 hours. In 12 animals synovial fluid was sampled from the affected joint under light thiopentone anaesthesia and the cavity washed twice with 2 ml of sterile saline. Samples were extracted and bioassayed for PG-like activity (Gilmore, Vane & Wyllie, 1968). In 10 out of 12 animals PG-like activity (range 4-51 ng PGE_2-equivalents) was detected in the synovial fluid.

In 3 animals indomethacin (200 μg/kg dissolved in phosphate buffer 0.1 M pH 8.9) was injected in the knee joint at the moment of the provocative injection of endotoxin: no symptoms developed in these animals up to 7 h after the injection.

An intravenous injection with endotoxin 24 h after a preparatory injection with concanavalin A (Sigma; 0.1-1 mg/kg, 8 experiments) or lipid A/BSA (supplied by Dr C. Galanos; 5 μg/kg, 2 experiments) gave incapacitation and production of PG's in all animals studied.

These experiments show that a Shwartzman-like reaction can be produced in the knee joint of the dog, during which PG's are released and which can be prevented by local treatment with indomethacin. These findings indicate that PG's may be involved in the local Shwartzman reaction. Furthermore, we think this to be a useful and practical model to study the release of chemical mediators and cells during the local Shwartzman reaction.

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These experiments were performed with the aid of the Fund for Collective Fundamental Research, Belgium. We thank Dr J.E. Pike, of Upjohn, Kalamazoo, for the prostaglandins, Dr C. Galanos, Freiburg (Br)-Zähringen, for lipid A/BSA and Merck, Sharp and Dohme, Belgium, for indomethacin.

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Gastric acidity and the mechanisms by which prostaglandins prevent indomethacin-induced gastric erosions

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Antisecretory prostaglandins inhibit the formation of gastric ulcers and erosions induced by several techniques (Robert, Nezamis & Phillips, 1968). In the present study, the relationship between inhibition of gastric acid secretion and the prevention of indomethacin-induced mucosal erosions by several prostaglandins has been investigated in the rat.

Indomethacin (5-30 mg/kg s.c.) caused the formation of rat gastric mucosal erosions within 1-6 h, the incidence and severity depending on the dose and duration of administration. Prostaglandin E_2 (PGE_2) and its methyl analogues, which are potent inhibitors of rat gastric secretion (Main & Whittle, 1975), reduced erosion formation in a dose and time-dependent manner. The dose reducing the erosion 'score' by 50% (ED_{50}) observed 3 h after indomethacin (15 mg/kg s.c.), was 220, 250, 0.54 and 0.50 μg/kg s.c. for PGE_2, PGA_2, (15S)-15 methyl E_2 and 16, 16, dimethyl E_2 respectively. However, the H_2-receptor antagonist metiamide, in doses which inhibited gastric secretion, also reduced indomethacin erosions; the ED_{50} was 13 mg/kg s.c. The ratio of the ED_{50} for prevention of erosions to the ED_{50} for inhibition of gastric acid secretion in the chronic fistula rat (see Main & Whittle, 1975) was 1.6 for PGE_2 and 0.4 for its methyl analogues, showing a variable relationship. Furthermore, the (15S) methyl analogue of $PGF_{2\alpha}$ also prevented erosion formation (ED_{50} 20 μg/kg s.c.).

The mechanism by which prostaglandins inhibit erosions was further studied by perfusing acidic saline (pH 1-2; 100-10 mM HCl) through the gastric lumen of the urethane-anaesthetized rat. Indomethacin (5-20 mg/kg s.c.) induced a low incidence of erosion formation within 3 h at pH 1.2-2.0, but this effect was greatly potentiated, accompanied by mucus secretion, when the bile salt sodium taurocholate (0.2-5.0 mg/ml) was perfused simultaneously. The time-course and degree of erosion formation, which was followed more closely by enclosing the exteriorized stomach in a transparent chamber (Mersereau & Hinchey, 1973), depended on the acid and taurocholate concentrations and the dose of indomethacin. Administration of the PGE_2 methyl analogues (5 $\mu g\ kg^{-1}\ h^{-1}$ s.c.) prevented indomethacin (20 mg/kg s.c.)-induced erosions during gastric perfusion of taurocholate (1 mg/ml; 2 mM) at pH 1.3 (76 ± 9% inhibition of erosions, n = 4; mean ± s.e.mean) and pH 1 (53 ± 12%, n = 4) for 3 h, and the mucosa appeared hyperaemic. The loss of titratable acid from the gastric lumen, which followed indomethacin administration with taurocholate and acid perfusion, was also reduced by the PGE_2 methyl analogues (20 ± 5% reduction, n = 8).

These results indicate that the formation of indomethacin-induced gastric erosions in the rat depends on the acid concentration in the gastric lumen, and that antisecretory agents such as prostaglandins and metiamide can inhibit their formation. However, the ability of prostaglandins to prevent erosion formation in the presence of exogenous acid indicates that other mechanisms may also be involved. It is not yet known how such mechanisms are related to changes in local blood flow or mucosal permeability.

This work was supported by the M.R.C. The supply of prostaglandins from the Upjohn Company, Kalamazoo, and metiamide from Smith, Kline and French Ltd. is gratefully acknowledged.

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The effect of prostaglandin E_1 and E_2 on drug-induced release of $[^3H]$-noradrenaline from rat mesenteric arteries

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The release of $[^3H]$-noradrenaline ($[^3H]$-NA) from sympathetic nerve endings by nerve stimulation is inhibited in the presence of prostaglandin E_1 and E_2 (Hedqvist, 1973). Prostaglandin E_1 and E_2 also inhibit the action of noradrenaline on a number of tissues (Bergstrom, Carlson & Weeks, 1968).

To investigate the effects of prostaglandins E_1 & E_2 on ^{3}HNA release by sympathomimetics, the rat mesenteric artery preparation was perfused with (–)-noradrenaline-$[7\text{-}^3H]$ and carrier (–)-noradrenaline diluted with normal Krebs to give a final concentration of 4.2×10^{-9} Ci/ml $[^3H]$-(–)-noradrenaline and 200 ng/ml noradrenaline respectively as previously described (George & Leach, 1972). The mesentery was perfused with this solution for 60 min after which it was then perfused with normal, tracer free Krebs solution and the spontaneous release of $[^3H]$-NA measured over a further 60 min period as described, (George & Leach, 1975). The effect, of perfusion with prostaglandin E_1 and E_2 (1-100 ng/ml) on the spontaneous release of $[^3H]$-NA was measured. Finally, the effects of perfusion with prostaglandin E_1 and E_2 on $[^3H]$-NA release caused by injection of NA 200 ng, metaraminol 20 µg, octopamine 50 µg and tyramine 100 µg were studied and the results for prostaglandin E_1 are summarized in Table 1.

These results show that prostaglandin E_1 at each of the perfusion concentrations used has an inhibitory effect on the release of $[^3H]$-NA by sympathomimetic amines but no effect on the spontaneous release of $[^3H]$-NA in the absence of the sympathomimetic amines. Prostaglandin E_2 caused a similar though smaller inhibition. It appears that prostaglandins of the E series could have some role in the drug induced release of $[^3H]$-NA from sympathetic nerve endings.

Table 1 The mean increase in ^{3}H-noradrenaline outflow ± s.e. following injection of each of the amines during perfusion with normal Krebs in the presence or absence of prostaglandin.

		Total mean increase in $[^3H]$-noradrenaline outflow ($d\ min^{-1}\ ml^{-1}$) ± s.e. following injection of each of the amines in the presence or absence of prostaglandin			
Perfusate composition	*Mean spontaneous ^{3}HNA release $d\ min^{-1}\ ml^{-1}$*	*Noradrenaline 200 ng*	*Octopamine 50 µg*	*Metaraminol 20 µg*	*Tyramine 100 µg*
Normal	1901	920 ± 101	2370 ± 210	3261 ± 332	2522 ± 241
Prostaglandin E_1 1ng/ml	2004	877 ± 88	1782 ± 177	2817 ± 226	1593 ± 184
Prostaglandin E_1 10 ng/ml	1986	735 ± 72	1211 ± 120	1872 ± 163	1009 ± 116
Prostaglandin E_1 100 ng/ml	1899	710 ± 73	1089 ± 110	1377 ± 124	725 ± 93

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Possible contribution of prostaglandins to genetic hypertension in rats: identification of a biochemical lesion

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Prostaglandins (PGs) E_2 and A_2, and the PGE_2 precursor, arachidonic acid, produce vasoconstriction in isolated perfused kidneys of normotensive rats (Malik & McGiff, 1975). PGE_2 is also vasoconstrictor *in vivo* as shown by experiments in which renal blood flow was measured with an electromagnetic flow probe in pithed or anaesthetised rats. Intravenous or intra-arterial PGE_2 (100-3000 ng) reduced renal blood flow (from approximately 10 ml/min to 2 ml/min) whereas acetylcholine bromide (500 ng, i.v.) caused an increase (to 14 ml/min).

The kidney may play a pivotal role in the pathogenesis of hypertension (Guyton & Coleman, 1969). Therefore, we sought possible disturbances of renal prostaglandin mechanisms in male genetic hypertensive rats (indirect systolic blood pressures 160-190 mm Hg) of the New Zealand strain, using Charles River Wistar normotensives (110-130 mm Hg), matched for sex and either age or weight as controls. Both kidneys of each animal were perfused *in vitro* with Krebs solution (gassed with 95% O_2 and 5% CO_2) at 37°C via an aortic cannula (10 ml/min per kidney). The renal effluent was continuously assayed for PG-like activity using the superfused organ technique of Ferreira & Vane (1967). Changes in mean perfusion pressure were measured using a transducer connected to the system proximal to the kidneys.

PGE-like activity, indicated by contraction of chick rectum, was detected in the renal perfusate concomittantly with the pressor responses which followed intra-arterial injections of either (−)noradrenaline bitartrate (200-1600 ng) or angiotensin II amide (5-80 ng). Intra-arterial infusions of indomethacin (1-2 μg/ml) prevented the appearance of the PGE-like activity and reduced the magnitude and duration of the vasoconstriction caused by the pressor agents. Thereafter, potentiation of the pressor effects of noradrenaline occurred when PGE_2 was infused into the kidneys in amounts (100-300 pg/ml) which by themselves had no pressor activity. The renal vasculature of hypertensives released greater amounts of PGE-like activity following intra-arterial vasoconstrictor agents than normotensives of the same age but this difference was not found when kidneys of equal weight were used.

We have also compared in normotensive and hypertensive animals the activity of the enzymes responsible for synthesizing or metabolizing PGs. There was a considerably decreased activity of 15-hydroxyprostaglandin dehydrogenase in the kidneys of genetic hypertensive rats (76.2 ± 2.9 fmol PGE_2 oxidized/mg protein per min, $n = 5$) relative to normotensive animals of similar weight (201.2 ± 3.3 fmol PGE_2 oxidized/mg protein per min, $n = 5$). The difference was also observed when both groups were matched for age. There was no difference between the prostaglandin biosynthetic capacities of hypertensive and normotensive kidneys.

Thus, higher than normal concentrations of PGE_2 may be present in the kidneys of genetic hypertensive rats. As small amounts of PGE_2 potentiate the vasoconstrictor effects of noradrenaline and larger quantities cause vasoconstriction, increased renal levels of the prostaglandin could contribute to the maintenance of the elevated blood pressure. The deficiency in prostaglandin dehydrogenase could be the primary genetic lesion responsible for the development of genetic hypertension in these rats.

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Effects of ketamine on the peripheral sympathetic nervous system of the rat

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Ketamine (2-(*o*-chlorophenyl) 2-methylamino cyclohexanone) is unique among the intravenous anaesthetics in elevating arterial pressure in man and animals (Virtue, Alanis, Mori, Lafargue, Vogel & Metcalf, 1967). One mechanism suggested to account for this pressor effect is blockade of neuronal uptake of noradrenaline (NA) leading to potentiation of the effects of sympathetic nerve activity. Studies indicating this action, however, involved high concentrations of ketamine *in vitro* or continuous infusion *in vivo* (Nedergaard, 1973; Montel, Starke, Gorlitz & Schumann, 1973). We have, therefore, compared the effects of a wide range of doses of ketamine both *in vitro* on the rat anococcygeus muscle and *in vivo* on the pithed rat arterial pressure and anococcygeus. The *in vivo* experiments were on rats pithed by the method of Gillespie, MacLaren & Pollock (1970). Carotid arterial pressure and anococcygeus tension were recorded. Preganglionic sympathetic nerves to blood vessels and to the anococcygeus were stimulated via the pithing rod electrode at L1-2; postganglionic sympathetic nerves to the anococcygeus at S1-S2 (Gillespie & McGrath, 1973). The *in vitro* preparation of the anococcygeus was set up according to Gillespie (1972).

In the pithed rat, ketamine (2-50 mg/kg) initially lowered, then raised arterial pressure. Pressor responses to stimulation at L1-2 (10 Hz, 10 s) were inhibited at doses above 2 mg/kg whereas those to NA (400 ng/kg) were not significantly affected. Ketamine did not change anococcygeus resting tension, but potentiated its motor response to NA at doses above 10 mg/kg and inhibited its responses to pre- and post-ganglionic stimulation; inhibition of responses to pre-ganglionic stimulation was greater.

In the anococcygeus *in vitro*, ketamine (3.7×10^{-7}-3.7×10^{-3} M) produced a dose-related inhibition of responses to carbachol, NA and field stimulation. The inhibition of NA responses was significantly less than that of carbachol responses in the range 3.7×10^{-5}-3.7×10^{-4} M. Cocaine (10^{-7}-10^{-5} M) potentiated responses to NA and field stimulation without affecting responses to carbachol; cocaine 10^{-4} M depressed responses to carbachol and field stimulation.

The results in the anococcygeus confirm that the sensitivity of smooth muscle to NA can be potentiated by high doses of ketamine (Nedergaard, 1973; Montel, Starke, Gorlitz & Schumann, 1973) although to a much lesser extent than by cocaine. At such doses, however, ketamine also possesses a non-specific depressant action which can produce ganglionic blockade and post-synaptic depression and would contribute only to a depressor effect.

This suggests that in the doses used clinically or in intact animals, ketamine owes its effect on arterial pressure to no single effect such as block of neuronal NA uptake and that the vasopressor response is not peripherally mediated.

A.S.C. is in receipt of a Wellcome Trust Scholarship and J.C.McG. of a Wellcome Trust Interdisciplinary Fellowship.

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Cardiovascular responses of the anaesthetized cat to lower body negative pressure

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By enclosing the lower limbs and pelvis of human subjects in an airtight box these parts may be exposed to pressures below atmospheric This manoeuvre—lower body negative pressure (L.B.N.P.)—causes blood to pool in the parts exposed to the suction and evokes compensatory reflex responses. This is a non-invasive procedure which has been used to investigate the effect of drugs on autonomic mechanisms in man (Ardill, Bhatnagar & Fentem, 1967; Bannister, Ardill & Fentem, 1969). The method has recently been extended to the investigation of cardiovascular control in the rabbit (Yates & Fentem, 1975) and some of the findings were sufficiently different from those reported in man to prompt a similar investigation in the cat.

Measurements of systemic arterial blood pressure, heart rate, and, in some animals, cardiac output by thermodilution were made on cats anaesthetized with chloralose 70 mg kg^{-1}. When L.B.N.P. was applied for 1 min at −70 to −85 mmHg, the arterial blood pressure showed a transient fall of 22 ± 4.4 mmHg ($n = 5$) but returned to resting levels within 30 s even though suction was still being applied. Cardiac output at 30 sec had fallen by 26%; the change in heart rate was variable (+28 to −32 beats min^{-1}). There was an increase in total peripheral resistance. Thus the stimulus induced compensatory changes in these control cats which, in the absence of drugs, were unchanged over a 7 h period of observation ($n = 4$) and were proportional to the degree of L.B.N.P. During 2 h following a single dose of bethanidine (0.3 mg kg^{-1} i.v., $n = 5$) there was a fall in resting arterial pressure of 18 ± 4 mmHg and with L.B.N.P. (−70 to −85 mmHg) a further fall in arterial blood pressure occurred (29 ± 3.6 mmHg) which persisted for the whole period of suction. No increase in total peripheral resistance occurred, and there was a persistent bradycardia (-23 ± 4 9 beats min^{-1}).

After a single dose of thymoxamine of 0.5 mg kg^{-1} i.v. ($n = 4$) maintained by an infusion of 0.5 mg $kg^{-1}h^{-1}$ the resting arterial blood pressure fell by 28 ± 8 mmHg and again with L.B.N.P. there was a further fall of 17 ± 5.1 which persisted for the duration of the suction, two cats had an increase in heart rate of 10 beats min^{-1}, two showed no change.

Thus L.B.N.P. in the cat induced reflex cardiovascular changes which were modified by a noradrenergic neurone blocking agent and by an α-receptor blocking agent. This technique would appear to be potentially useful in the analysis of drug mechanisms in cardiovascular control in anaesthetized animals.

This work was supported by a research grant from the M.R.C. to P.H.F.

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Mechanism of K$^+$-induced release of cytoplasmic noradrenaline from adrenergic nerves

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Wakade and Kirpekar (1974) reported that K$^+$ released noradrenaline (NA) from reserpine-pretreated and MAO-inhibited vasa deferentia of guinea pigs by a Ca^{++}-independent process. In the present study, the mechanism of this effect has been further examined.

As described previously (Paton, 1973), atria, from reserpine-pretreated rabbits, were exposed to pargyline (5×10^{-4} M for 30 min) and tropolone (10^{-4} M), and then to 5×10^{-7} M [^{3}H]-(−)-NA for 60 minutes. Tissues were blotted and transferred every 5-10 min to fresh media at 37°C. Tissues were exposed to elevated K$^+$ for 40 min after 50 min of efflux. At 90 min tissues were removed and their ^{3}H content determined. Media had the following composition: (a) control medium (mM) NaCl, 140; KCl, 5.0, $CaCl_2$, 1.5, $MgCl_2$, 1.2; Tris-HCl, 20, and (b) medium with elevated K$^+$: as above except NaCl, 100, KCl, 65.

Efflux of [^{3}H]-NA was not accelerated by 25 mM KCl. However, 45-85 mM KCl caused a progressive increase in [^{3}H]-NA efflux. In subsequent studies, the effects of 65 mM K$^+$ were examined. Efflux was also accelerated by 65 mM RbCl and CsCl, the relative potencies being $K^+ = Rb^+ > Cs^+$.

The accelerative effect of 65 mM KCl on [^{3}H]-NA efflux was not altered by 10^{-5} M phentolamine, 10^{-7} M oxymetazoline, 5.6×10^{-5} M indomethacin, 10^{-4} M procaine or 10^{-4} M lidocaine, but was significantly inhibited by 1.5×10^{-5} M cocaine and 10^{-6} M desipramine, and was abolished when tissues were loaded with [^{3}H]-NA in the presence of 3×10^{-5} M cocaine, 10^{-4} M hydrocortisone and 10^{-4} M oxytetracycline.

K$^+$-induced release of NA from untreated tissue (i.e., with normal vesicular storage of NA) is Ca^{++}-dependent and is subject to α-receptor feedback inhibition (Kirpekar and Wakade, 1968; Stjärne, 1973; Starke & Montel, 1974). By contrast, this study and that of Wakade and Kirpekar (1974) has shown that elevated K$^+$ accelerates the efflux of [^{3}H]-(−)-NA from extragranular, cytoplasmic sites in peripheral adrenergic nerves by a process that is not subject to α-receptor or prostaglandin-mediated feedback inhibition, does not require Ca^{++}, and is inhibited by cocaine and desipramine. These findings show that the process does not result from exocytosis of NA. It is suggested that raising the external K$^+$ concentration caused membrane depolarization and that this accelerated the loss of NA via a cocaine- and desipramine-sensitive carrier. Ouabain, omission of external K$^+$ and metabolic inhibition also accelerated the efflux of [^{3}H]-NA and these effects were all inhibited by cocaine (Paton, 1973). These procedures also produce membrane depolarization. It has been proposed that the membrane potential is also involved in the uptake (or influx) of NA in adrenergic nerves (Holz, Deguchi & Axelrod, 1974, White, 1975).

Supported by grants from the Medical Research Council of Canada (MT 2474) and the Alberta Heart Foundation.

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The time course of noradrenaline release caused by high potassium-low sodium solution. Effects of methacholine or decrease of the calcium ion concentration.

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Muscarinic drugs including methacholine (MCh) have previously been shown to inhibit calcium-dependent release of noradrenaline by various stimuli (Muscholl, 1973). The present report deals with two phases of noradrenaline release caused by high K^+-low Na^+ solution (equivalent substitution of 132.3 mmol/l NaCl of Tyrode solution by KCl) and the selective effects of MCh or calcium deprivation on the first phase.

Hearts were removed from rabbits (1.6-2.1 kg) and perfused at constant flow of 20 ml/min according to the Langendorff method with Tyrode solution at 36.5°C. After 20 min the perfusion with high K^+-low Na^+ was started. Noradrenaline in the perfusates was determined fluorimetrically and the rate of output plotted on semi-log paper.

The noradrenaline output of the hearts during perfusion with high K^+-low Na^+ declined exponentially in two phases with half-times of 1.59 and 16.9 min (Table 1). The initial rates of release differed by a factor of 10. The first phase did not extend beyond the 10th minute. The total noradrenaline output from 0-10 min was 3 times that from 10-30 min, the latter comprising only phase two.

MCh was perfused 0.5-15 min before and during high K^+-low Na^+. Independent of the *duration* of pre-perfusion, but depending on the *concentration*, MCh decreased the initial rate of noradrenaline release and its total output from 0-10 minutes. However, the second phase of noradrenaline release remained unaffected by MCh. Atropine (1.44 μmol/l) did not alter the above parameters but completely antagonized the effects of MCh (320 μmol/l). When the calcium ion concentration of high K^+-low Na^+ solution was reduced from 1.8-0.113 mmol/l the semi-log plot of outputs could not be resolved into two phases. The output 0-10 min depended on the length of pre-perfusion of low calcium solution. After 1, 3, 30 and 60 min the outputs were 1085 ± 153; 839 ± 180; 537 ± 114 and 393 ± 63 ng, respectively (each n = 3). In contrast, the outputs from 10-30 min were not significantly different from that observed in normal calcium solution.

Table 1 Effect of MCh on the rate of noradrenaline output evoked by high K^+-low Na^+

MCh μmol/l	*n*	*First phase*		*Second phase*		*Total noradrenaline released*	
		Initial rate (ng/min)	*$T_{½}$ (min)*	*Initial rate (ng/min)*	*$T_{½}$ (min)*	*0-10 min (ng)*	*10-30 min (ng)*
0	8	1240 ± 115	1.59 ± 0.18	124 ± 10.9	16.9 ± 2.2	3254 ± 315	1038 ± 43
40	6	373** ± 110	2.11 ± 0.55	144 ± 21	16.4 ± 1.4	1741* ± 284	1050 ± 187
320	12	228** ± 32	2.88** ± 0.25	122 ± 10.4	17.5 ± 1.0	1457** ± 122	1119 ± 113

Given are means ± s.e. of *n* observations. Level of significance (*t*-test) from values of first line,
* $P < 0.05$; ** $P < 0.005$.

The findings confirm the suggestion that excitation of muscarinic receptors decreases the availability of calcium for noradrenaline release.

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Stimulation and inhibition by ouabain of the sodium pump in guinea-pig atria

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Studies on isolated human heart preparations have shown that, within the range of free therapeutic concentrations ($\pm 3 \times 10^{-9}$ M), digitoxin did not inhibit the sodium pump but stimulated ^{42}K uptake (Godfraind, 1973). It has also been reported that low ouabain concentrations (10^{-9} M) stimulated ^{42}K pumping in guinea-pig atria and produced a supersensitivity to catecholamines (Godfraind & Godfraind-De Becker, 1965; Godfraind & Lesne, 1972). The present experiments, performed on electrically stimulated left guinea-pig atria, were designed in order to see whether the actions of therapeutic concentrations might be related to the binding of ouabain to the cardiac glycoside receptors responsible for the inhibition of the sodium pump.

Isolated left guinea-pig atria were bathed at 30°C in Tyrode solution (mM : NaCl 137, KCl 6 instead of 2.7, $CaCl_2$ 1.82, $MgCl_2$ 0.105, NaH_2PO_4 0.417, $NaHCO_3$ 11.9, glucose 5.5) equilibrated with a mixture of 95% O_2/5% CO_2. The atria were electrically stimulated at a rate of 3.3 Hz. Their contractility was recorded by means of isometric levers (resting tension ± 500 mg). They were stimulated by cumulative increment of isoprenaline up to 10^{-5} M. After 30 min resting period, ouabain, at concentrations of between 10^{-9} and 10^{-4} M, was added for three hours. At the end of the experiment, the atria were mineralized and the ionic content was determined by atomic absorption spectroscopy. The binding of [^{3}H]-ouabain was estimated as previously reported (Godfraind & Lesne, 1972).

Low concentrations of ouabain (10^{-9} to 10^{-8} M) stimulated the sodium pump as shown by the increase of the K content associated with an equivalent decrease of the Na content (mmol/kg wet wt; controls, (15), Na . 74.8 ± 1; K: 63.9 ± 0.9; atria incubated with ouabain 3×10^{-9} M (8) Na: 70.1 ± 1.4, K: 68.4 ± 1.3; $P = 0.02$).

Higher concentrations inhibited the pump, a dose-dependent Ca gain associated with a Mg loss was noticed when the inhibition reached 30%.

The positive inotropic effect of ouabain occurred for concentrations stimulating the Na pump, it increased with the inhibition of the pump. The maximum increase in systolic tension was of 250%, the dose producing 50% of the maximum was 3×10^{-7} M. For ouabain concentrations lower than 3×10^{-7} M, the increase in systolic tension was maintained over the 3 h of the observation period. At higher concentrations, the increase in systolic tension reached an optimum and decreased with the increase of the diastolic tension.

Confirming earlier results, [^{3}H]-ouabain uptake showed a linear and a saturable component (Godfraind & Lesne, 1972). The latter was a Langmuir binding curve with a K_e of 4×10^{-7} M and approaching complete saturation at 3.4×10^{11} molecules of ouabain per mg wet wt, it intersected the dose-effect curve relating ouabain-sensitive K content to ouabain concentration near 50% of the maximum. This provides evidence that the saturable binding sites and the pumping sites are the same. A further evidence was provided by their $(K)_o$ dependency for a reduction of $(K)_o$ from 6 mM to 2.7 mM the Langmuir binding curve was displaced to the left with a K_e of 1.9×10^{-7} M and a complete saturation at 1.3×10^{11} molecules of ouabain per mg wet wt; the dose effect curve for the action of ouabain on the pump was displaced to the same extent, the dose producing 50% of the maximum inotropic effect was reduced to 4×10^{-8} M. Furthermore, the maximum increase in systolic tension was only of 80%, as if the inotropic effect was related to the number of ouabain molecules bound.

It has been reported that the contractility of the atria was dependent on $(Na)_i$ (Glitsch, Reuter & Scholz, 1970). The systolic tension and the Na content were respectively 34% and 10% lower for $(K)_o = 6$ mM than for $(K)_o = 2.7$ mM.

This indicates that the contractility was reduced by $(K)_0$ stimulation of the sodium pump.

As the pump stimulation by ouabain is associated with an increase in systolic tension, the interaction of the glycoside with the receptor probably involves supplementary changes.

This work has been supported by FRSM (grant n° 20.121). We thank Mr A. Goffin for technical assistance.

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An attempt to measure the lifetime of sodium channels in transporting epithelia

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Recently it has proved possible to use $[^{14}C]$-amiloride as a reversible site label for sodium entry sites in cells isolated from transporting epithelia (Cuthbert & Shum, 1975). This communication reports an attempt to determine the lifetime of sodium entry sites in isolated cells prepared from toad bladders (*Bufo marinus*). This parameter is of interest, particularly when considering the effects of aldosterone, which we have shown previously causes an increase in the number of binding sites (Cuthbert & Shum, 1975)

Suspensions of epithelial cells were prepared as described previously and binding of amiloride measured at a concentration of 50 nM. At this concentration non-specific binding accounts for only 20% of the total displaceable label.

In initial experiments cells were suspended in a nutrient salt solution (containing 1.1 mEq/l Na^+) with penicillin G (1000 u/ml) and streptomycin (0.5 mg/ml) and incubated with gentle shaking at room temperature for periods of up to 144 hours. Periodically aliquots were withdrawn for labelling. The slopes of the regression lines relating the number of binding sites with respect to time were not significantly different from zero. In other experiments cycloheximide (0.5 μg/ml) was added to the cell suspension at about 6 h and aliquots were withdrawn for labelling as before. The times taken for the binding site density to fall to half the original values were 60, 57, 32 and 61 h in four separate experiments.

Cell suspensions which had been exposed to aldosterone (50 nM) for 4 h were then treated with cycloheximide (0.5 μg/ml) and labelled with amiloride. Compared with control suspensions derived from the same tissues, but not exposed to aldosterone, there was a significant ($p < 0.001$) increase in the number of amiloride binding sites. The mean increase in 5 experiments was 50%.

The time course of the decline in the density of binding sites in aldosterone and cycloheximide treated suspensions was identical to that of non-hormone treated cells, suggesting that pre-existing and aldosterone induced sites have similar lifetimes. Provided that amiloride binding sites can be equated with sodium entry sites then this result has consequences for the duration of the aldosterone response, provided sodium entry and not other energetic factors (see for example Sharp & Leaf, 1966) remains the rate determinant of transport.

This work was supported by a grant from the M.R.C.

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DL-Homocysteate-induced motoneurone depolarization with membrane conductance *decrease*

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The non-physiological ion DL-homocysteate (DLH), applied by microiontophoresis, has been used widely to excite cells for extracellular recording in neuropharmacological studies. It has been of particular value for investigations of putative inhibitory transmitters (Curtis, 1965; Engberg & Ryall, 1966). It has been supposed that the action of DLH and other excitatory amino acids is accompanied by an increase in membrane conductance (Curtis, 1970, Curtis, Duggan, Felix, Johnston, Tebecis & Watkins, 1972). We have shown that the DLH induced depolarization of motoneurones is accompanied by a *decrease* of conductance.

DLH 0.3 M, L-glutamate 1 M, L-aspartate 1 M, (all at pH 8) were applied from the six iontophoretic barrels of a coaxial electrode assembly to lumbar motoneurones of seven cats (four pentobarbitone, three decerebrate).

Membrane conductance (G_m) was measured by passing current pulses through the screened central recording electrode.

Typical responses to DLH are shown in Figure 1.

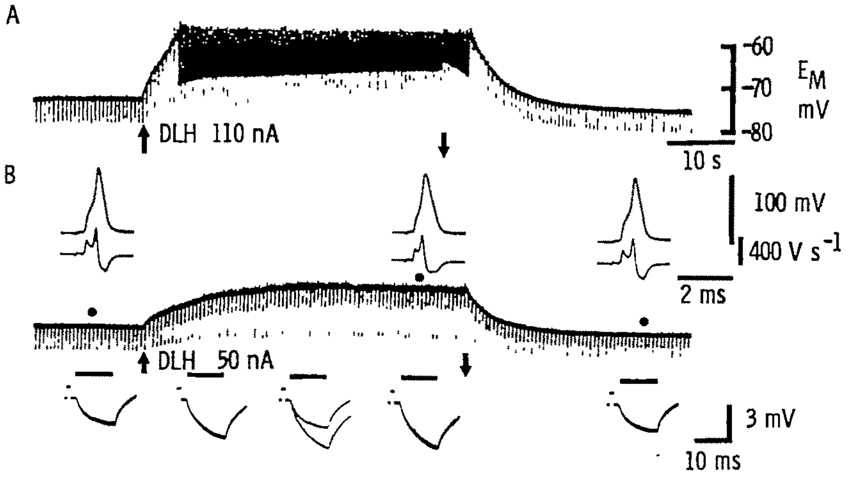

Figure 1 Responses of a hamstring motoneurone to iontophoresed DLH in a nembutal anaesthetized cat The membrane potential (E_m) was displayed on a mingograph—the traces being modulated by the after-hyperpolarizations of antidromically evoked or DLH induced action potentials and by short hyperpolarizing current pulses. *A*. An unbalanced dose (110 nA) of DLH reversibly depolarized the cell At 15 mV depolarization the cell fired repetitively at a high frequency. *B*. A lower dose (50 nA) of DLH caused a depolarization of 10 mV, insufficient to evoke firing. Conductance measuring pulses (3 nA, 10 ms) are shown below and were averaged during the periods shown by the bars. The conductance pulse recorded before DLH application is superimposed on the pulse recorded during DLH application in the middle record. Antidromic action potentials and their first derivatives were recorded at the times indicated by the filled circles.

DLH reversibly depolarized the cell which led to repetitive firing with little adaptation (Figure 1A). At a dose insufficient to cause firing the DLH depolarization was accompanied by a marked decrease in G_m (Figure 1B). During the DLH induced depolarization the excitatory post-synaptic potentials (e.p.s.ps) changed differently from neurone to neurone: an increase in amplitude; an increase in the time constant of decay; a combination of these changes or, less commonly, no change. Both inhibitory post-synaptic potentials (i.p.s.ps) and after-hyperpolarizations showed stable increases in size.

Glutamate at low doses caused a depolarization accompanied by either: a small decrease, no change or a small increase in G_m. At higher doses glutamate produced a plateau of depolarization during which the conductance gradually rose until the membrane was virtually short-circuited—as has been similarly seen by Zieglgängsberger & Puil, 1973. Glutamate has previously been seen to depolarize with little or no change in G_m (Bernardi, Zieglgängsberger, Herz & Puil, 1972; Steinberg, Altmann & ten Bruggencate, 1974). The results from one cat indicate that aspartate essentially acts in the same way as glutamate.

J.D.C.L. gratefully acknowledges a travelling grant from the Wellcome trust.

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Synaptic vesicle magnesium-adenosine triphosphatase as a possible site of action of anticonvulsant drugs

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Phenytoin and ethosuximide inhibit a magnesium-activated adenosine triphosphatase (Mg-ATPase) located in the vesicle-containing fraction derived from nerve terminals prepared from rat cerebral cortex (Gilbert & Wyllie, 1974a,b). Mg-ATPase activities in the membrane and mitochondrial fractions derived from the nerve terminals were not influenced by the drugs.

We have recently tested the effects of diazepam and the anticonvulsants phenobarbitone, trimethadione, acetazolamide, sodium valproate and SC 13504 (Searle) on the Mg-ATPase activity of synaptic vesicles using techniques similar to those used previously to prepare synaptosomes, to disrupt them by osmotic shock, to recover the sub-synaptosomal fractions and to assay ATPase activities (Gilbert & Wyllie, 1974a,b; 1975).

All the anticonvulsants and diazepam, when added to preparations *in vitro*, inhibited the Mg-ATPase contained in the fraction enriched in synaptic vesicles but not the enzyme contained in the other fractions prepared from the synaptosomes. Other drugs tested, including convulsants, did not inhibit the enzyme in the vesicle fraction. Administration of phenytoin (50 mg/kg and 100 mg/kg) or phenobarbitone (25 mg/kg and 100 mg/kg) *in vivo* to rats and subsequent preparation of the vesicle-containing fractions showed that the Mg-ATPase was inhibited to the maximum degree which had been observed in the experiments involving the addition of the drugs *in vitro*.

Dose response curves indicated that the relative potencies of the anticonvulsants with regard to inhibition of the enzyme were compatible with their relative potencies in preventing seizure activity in mice.

Studies of the distribution of phenytoin using the assay technique of Dill & Glazko (1972) suggested that the drug was present in the synaptosome cytoplasm and vesicle fractions.

This work suggests that the Mg-ATPase of nerve terminal vesicles may be implicated in the mechanism of action of anticonvulsants.

We wish to thank Parke-Davis & Co. for financial support and for supplying phenytoin and ethosuximide. We thank G.D. Searle & Co. Ltd., Reckitt & Colman and Abbott Laboratories Ltd. for supplying SC 13504, sodium valproate and trimethadione respectively.

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Interaction of carbenoxolone sodium with other drugs bound to plasma proteins

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Displacement from plasma protein binding sites has been suggested as one of the causative factors in increased efficacy or toxicity during concomitant administration of two or more drugs (O'Reilly & Levy, 1970; Mason & McQueen, 1974; Pugh, Geddes & Yeoman, 1975). Carbenoxolone sodium is an ulcer-healing drug which is highly bound to plasma proteins. Its efficacy, mode of action and low toxicity may even depend upon this binding (Parke & Lindup, 1973).

We have carried out a series of *in vitro* experiments with the fluorometric probe 1-anilino-8-naphthalene sulphonic acid using the method of Jun, Luzzi & Hsu (1972). Carbenoxolone sodium was studied together with several other protein-bound drugs which may be clinically encountered in carbenoxolone therapy. These included, phenylbutazone, flufenamic acid, prednisolone, aspirin, warfarin, tolbutamide, phenytoin and imipramine. The parent triterpenoid moeity of carbenoxolone, namely enoxolone, and an analogue, cicloxolone were also included. To enable comparison with other workers we used both crystalline bovine (BSA) and human (HSA) serum albumin solutions (Jun, *et al*, 1972; Mason & McQueen, 1974).

Initially we found a difference in binding potential between HSA and BSA. With BSA only one class of binding site was seen for the probe whereas two classes were seen with HSA. Moreover, carbenoxolone and phenylbutazone were both bound at the same class of site to BSA but at different classes of sites to HSA. For the remainder of the study HSA was used.

The binding characteristics of HSA of the drugs used formed three distinct groups;

Group 1 consisted of drugs with strong binding affinities to the class I site and included the three triterpenoids of which carbenoxolone was the most strongly bound.

Group 2 also included strongly bound drugs, but these were binding at the class II sites. Of these flufenamic acid was the most strongly bound, followed by phenylbutazone warfarin and tolbutamide, respectively.

Group 3 comprised the weakly bound drugs binding at both class I and II sites. Of these aspirin was the most strongly bound, followed by phenytoin, prednisolone, and imipramine, respectively.

Thus it is probable that interaction, due to displacement from binding sites, between drugs of groups 1 and 2 is unlikely. The weak binding affinities, to both classes of binding sites, of drugs in group 3 indicate that they may be susceptible to displacement by the drugs in both groups 1 and 2. Within each of the three groups of drugs, displacement may be expected of the drugs with the lowest binding affinities by those with a higher affinity within the same group.

Further studies are in progress designed to examine the specificity of carbenoxolone binding and to examine the indicated possible *in vivo* interactions.

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Tryptophan metabolism by the isolated perfused rat liver

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Increased rat liver tryptophan pyrrolase activity following hydrocortisone (5 mg/kg) injection decreases tryptophan concentrations in liver and brain and is accompanied by decreased brain 5-hydroxytryptamine synthesis (Green, Woods, Knott & Curzon, 1975). In this and other experiments investigating the effect of drugs on pyrrolase activity the enzyme has been measured *in vitro* following prior treatment of the animals *in vivo*. In an attempt to observe whether these *in vitro* observations have any relevance to the activity of the enzyme *in vivo*, we have investigated the effect of certain drugs on the activity of the enzyme in the isolated perfused rat liver. The concentration of kynurenine in the medium has been used as an index of enzyme activity. A small lobe of liver was also removed at the beginning and end of the perfusion and enzyme activity measured *in vitro*.

Rat livers were perfused by the method of Hems, Ross, Berry & Krebs (1966) with a semi-synthetic medium (Woods, Eggleston & Krebs, 1970); *in vitro* enzyme activity was measured as described by Curzon & Green (1969).

Perfusion of the basal medium without the addition of tryptophan resulted in a small amount of kynurenine appearing in the medium at the end of a 60 min perfusion (2.0 ± 0.1 μg kynurenine/g liver (wet weight)). The concentration of tryptophan in the basal medium was 0.84 ± 0.15 μg tryptophan/ml and rose linearly during the perfusion to reach a concentration of 4.6 ± 0.2 µg/ml at 60 minutes.

At the end of a 60 min perfusion and when 0.1 mM tryptophan was perfused the mean concentration of kynurenine was 6.64 µg/g liver (wet weight) and when 1.0 mM tryptophan was in the medium the final mean concentration of kynurenine in the medium was 31.1 µg/g liver. Pretreatment of the rats with hydrocortisone (5 mg/kg) 3 h before the start of the perfusion resulted in a threefold increase of pyrrolase activity as measured *in vitro* and increased the concentration of kynurenine in the medium at the end of the 60 min perfusion to 14.45 ± 2.82 µg/g when 0.1 mM tryptophan was perfused and 145 ± 13.3 µg/g when 1.0 mM tryptophan was perfused.

When allopurinol (20 mg/kg) was injected with the hydrocortisone (5 mg/kg) and also added to the 0.1 mM tryptophan medium (4 mg allopurinol /100 ml medium) it totally inhibited the hydrocortisone-induced rise in kynurenine concentration in the medium. The rise in pyrrolase activity (measured *in vitro*) produced by hydrocortisone injection was totally inhibited by the simultaneous injection of allopurinol (20 mg/kg).

The disappearance of tryptophan from the medium did not always reflect the activity of pyrrolase measured either *in vitro* or by the appearance of kynurenine in the medium. This is in agreement with the observations of Kim & Miller (1969). Unlike these authors, however, we find that hydrocortisone pretreatment increases subsequent kynurenine production by the isolated perfused rat liver. Furthermore, we observed that changes in pyrrolase activity measured *in vitro* were of the same order as the changes in kynurenine concentration in the perfusion medium.

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Role of platelet aggregation in bronchoconstriction in guinea-pigs

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Non-steroidal anti-inflammatory drugs (NSAID) prevent bronchoconstriction due to bradykinin (B) (Collier & Shorley, 1960), ATP and ADP (Collier, James & Schneider, 1966), arachidonic acid (AA) (Berry, 1966) and slow reacting substances (Berry & Collier, 1964; Vargaftig, Miranda & Lacoume, 1969). Since some of these agents cause platelet aggregation which is inhibited by NSAID, we tested the hypothesis that bronchoconstriction by B, by AA and by ADP or ATP are mediated by platelet release reaction occurring when clumps are trapped in lung vessels. This possibility was investigated in pentobarbitone anaesthetized guinea-pigs prepared for recording of bronchial resistance to inflation (Konzett-Rössler method) and of carotid blood pressure. Acetylcholine (5-20 µg kg^{-1} i.v.) was injected 3-5 times, until constant responses were obtained, after which other drugs were given. Platelet counts were made on arterial blood, and aggregation was studied by standard techniques (Born & Cross, 1963; Vargaftig, Tranier & Chignard, 1974) on guinea-pig platelet rich plasma obtained from citrated blood. Anti-platelet plasma (APP) was prepared by injecting thrice washed broken guinea-pig platelets in complete Freunds adjuvant into both fore paws of a rabbit. Two further s.c. injections were made at weekly intervals, blood was collected in citrate on the 21st day. APP lysed guinea-pig platelets placed on the aggregometer, which was inhibited by Na$_2$EDTA (2.5 mM) but unaffected by indomethacin (0.5 mM). Lyophilized APP was reconstituted to its initial volume with 0.9% NaCl (w/v) and injected slowly (1 h) i.v. into the guinea-pig (1 ml kg^{-1}).

Circulating platelets decreased 62 ± 4.8%

(mean ± s.e. mean) 10 s after ADP (100 μg kg^{-1}; n = 22) administration. One minute later the drop was 21 ± 7 and the count returned to basal levels within 5-10 minutes Higher doses of ATP (0.5-1 mg kg^{-1}) were required for similar effects. AA (500 μg kg^{-1}) decreased the number of platelets by 70% ± 4.4 (n = 11). Bronchoconstriction and the platelet decrease were suppressed by 10 mg kg^{-1} of aspirin i.v. B (0.5-1 μg/kg^{-1}) had no effect on platelet counts. Platelet depletion resulted in suppression of ADP- and of ATP-induced bronchoconstriction, whereas the effect of B and of AA was maintained or only slightly reduced. Administration of propranolol (5 mg/kg^{-1} + 2 mg/kg^{-1} i.v.) restored responses to B demonstrating that the reduction of its effect was due to antagonism by released adrenaline and not to platelet depletion (Collier, 1966). B failed to aggregate guinea-pig platelets in presence of the kininase inhibitor BPP9A (250 μg/ml^{-1}), whereas ATP induced a marked aggregation, probably via conversion to ADP. In conclusion, agents likely to start the release reaction and thus to activate prostaglandin synthesis (Zucker & Peterson, 1968; Smith, Ingerman, Kocsis & Silver, 1974) with formation of bronchoconstrictor substances (Vargaftig & Dao Hai, 1972a) interact with different sites, all of them subject to blockade by aspirin. In the case of ADP and of ATP, this site is circulating platelets. Bradykinin probably induces formation of bronchoconstrictor substances by activating release of prostaglandin precursors directly from the lungs (Vargaftig & Dao Hai, 1972b).

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The effect of sulphinpyrazone, sodium aspirin and oxprenolol on the formation of arterial platelet thrombi

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Platelets play a major role in the formation of arterial thrombi (see Mustard, Kinlough-Rathbone & Packham, 1974). In an effort to study the reaction *in vivo*, various methods of injuring the microcirculation have been examined to produce platelet thrombosis (white body formation) (see Didisheim, 1972).

In the present study we have used the hamster cheek pouch as described by Duling, Berne & Born (1968) and perfused with a modified Krebs solution at 5 ml/min as described by Duling & Staples (1974). The following parameters were found to be optimal for producing white body formation. After a 30 min equilibration period, a glass micropipette with a 3 μm tip filled with 1 MKCl was placed in contact at approximately 90° with the wall of an arteriole of 40-60 μm diameter. The solution in the micropipette was connected with a Ag/AgCl electrode via an external circuit to a reference Ag/AgCl electrode immersed in the outflow of the bathing solution A Bell stimulator in conjunction with a Devices isolated stimulator (type 2533) provided positive square wave DC pulses to the micropipette at 10 Hz, 20 ms and a current of 100 μA which were monitored by an oscilloscope in the circuit.

The amount of white body formation was assessed by counting, at a magnification of 350. the total thrombi adhering during the first 4 min after stimulation. A graded response could be produced by varying the duration of stimulation. Stimulation for 1, 2 or 4 s produced (mean ± s.e. mean (*n*)), 2.91 ± 0.54 (12), 3.8 ± 0.33 (17), 5.25 ± 0.49 (8) white bodies respectively. n refers to the number of 1, 2 or 4 s stimulations in six hamsters. The difference between the responses to 1 and 4 s were statistically significant ($P < 0.001$).

The method has so far been used to study the effects of sulphinpyrazone and aspirin which are being currently assessed in man as anti-thrombotic agents (Barnett, 1973). In addition, oxprenolol has been examined since β-adrenoceptor blockers have been reported to inhibit platelet aggregation (Bucker & Stucki, 1969).

Groups of 4-7 male hamsters, 100-120 g were given orally 18 h and 1 h before injury, one of the following treatments: 3, 10, 20 or 65 mg/kg of sulphinpyrazone (0.5 ml/100 g); 1, 10, 30, 60, 100 or 200 mg/kg of sodium aspirin (0.5 ml/100 g); or oxprenolol, 0.1, 1 or 5 mg/kg (0.2 ml/100 g).

Significant reduction in white body formation was found with all three drugs. Sulphinpyrazone produced a significant reduction for all periods of stimulation ($P < 0.001$ and $P < 0.05$) at 20 and 65 mg/kg respectively, a small reduction at 10 mg/kg and no effect at 3 mg/kg. Oxprenolol produced a significant reduction ($P < 0.05$) at 5 mg/kg but not at 1 mg/kg. Aspirin behaved differently in producing a bell-shaped dose response curve. Whereas 10 and 30 mg/kg produced a significant reduction ($P < 0.005$), both a lower dose (1 mg/kg) and higher doses (60 and 100 mg/kg) produced no significant reduction, while 200 mg/kg enhanced white body formation.

These results support the view that sulphinpyrazone is an effective anti-thrombotic agent and suggest a similar action for oxprenolol. However, the effectiveness of aspirin is variable according to dosage.

This work has been generously supported by CIBA-GEIGY.

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The effect of sulphinpyrazone on the thrombocytopenia occurring in the Arthus reaction

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Sulphinpyrazone is a uricosuric agent, which, *in vitro*, inhibits platelet aggregation and release caused by a large number of agents including collagen, ADP, antigen/antibody complexes, viruses and bacteria (Mustard & Packham, 1975). The drug has also shown, in a number of clinical trials, to have anti-thrombotic activity, and to reverse shortened platelet survival (Steele, Weily & Genton, 1973; Steele, Weily, Davies & Genton, 1974). However, some *in vitro* findings suggest that the *in vivo* effect of sulphinpyrazone may not be due to its direct interference with the aggregation of platelets (Mustard & Packham, 1975). In order to investigate further the problem of the mode of action of the drug, an examination of its influence on intravascular immune platelet aggregation and lysis occurring in the Arthus reaction in the rabbit has been commenced.

Rabbits were immunized using alum precipitated ovalbumin (1.0 mg, s.c.) 6 weeks prior to challenge with aqueous ovalbumin (10 μg or 1 mg) injected s.c. at each of six sites on the back. Thrombocytopenia, measured as a fall in the circulating level of homologous ^{51}Cr-labelled platelets injected (i.v.) 24 h earlier occurred acutely following antigenic challenge. Its magnitude varied with the antigenic dose, so that after 6 mg or 60 μg challenges, the radioactivity in whole blood fell to 24.5 ± 1.9% and 74.3 ± 6.7% of control value, respectively. Similar results were obtained by absolute cell counts but the variation between animals was larger. The thrombocytopenia persisted for 4 h after both doses of antigen. An obligatory role for complement was confirmed since depletion of complement to 10% of control level through the use of anti-complementary factor from Cobra venom was found to result not only in the abolition of the Arthus reaction but in the thrombocytopenia as well.

Sulphinpyrazone (50, and 30 mg/kg), administered as the sodium salt intravenously 1 h before challenge with 60 μg of antigen completely inhibited the thrombocytopenia. Upon challenging with 6 mg of antigen, sulphinpyrazone (50 mg/kg) restored the platelet count significantly ($P < 0.01$) to 44.5 ± 4.9% of control, but a dose of 30 mg/kg was not active. The inhibitory effects persisted for at least 4 h in all cases.

These results confirm that sulphinpyrazone can act relatively quickly to protect platelets against complement-mediated immune lysis in the Arthus reaction, an effect which is possibly analogous to its action in endotoxin shock (Evans & Mustard, 1968) and the Forsmann reaction (Tsai, Taichman, Pulver & Schönbaum, 1973). However, it remains to be established whether such an effect is due to a direct action on platelets or on the endothelium of the vasculature.

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Inhibition of 5-hydroxy-[^{3}H]-tryptamine binding to rat blood platelets by 5-HT antagonists and uptake inhibitors

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Rat blood platelets undergo a shape-change in response to sub-micromolar concentrations of 5-hydroxytryptamine (5-HT), and can also accumulate 5-HT against a high concentration gradient by active transport. We have recently shown that three [^{3}H]-5-HT binding sites exist on intact rat blood platelets (Drummond & Gordon, 1975), and in this present study we investigated the relationships between binding of the two highest affinity sites, production of the 5-HT-induced shape change and the active uptake of 5-HT. The experiments were performed with rat citrated platelet-rich plasma, and all the techniques used have been previously described in detail (Gordon & Drummond, 1974; Drummond & Gordon, 1975).

Table 1 Effects of 5-HT antagonists and uptake inhibitors on the high-affinity binding of [^{3}H]-5-HT to rat blood platelets and upon the platelet shape-change induced by 5-HT

	IC_{50} value (nM) against	
Compound	*5-HT-induced shape-change*	*[^{3}H]-5-HT binding at 4°C*
Pizotifen	1.2	1.3
D-LSD	5.5	1.2
Cyproheptadine	3.0	1.0
Cinanserin	2.8	1.4
Methysergide	5.5	2.0
Xylamidine	16.0	11.0
Chlorpromazine	32.0	24.0
Imipramine	250.0	100.0
Chlorimipramine	400.0	500.0
Lilly 110140 3-(p-trifluoromethylphenoxy)-N-methyl-3-phenylpropylamine	4000.0	700.0
Lilly 103947 3-(p-trifluoromethylphenoxy)-3-phenylpropylamine		

Specific binding of [^{3}H]-5-HT was essentially instantaneous and was directly proportional to the number of platelets Binding to the highest affinity site (Ka^{-1}= 10 nM) was prevented by 5-HT antagonists such as methergoline (IC_{50} = 0.7 nM). There was good correlation between inhibition of this [^{3}H]-5-HT binding and inhibition of the 5-HT-induced shape change (Table 1). Inhibitors of 5-HT uptake also affected shape change and binding to the highest affinity site, but only at micromolar concentrations. This provides direct evidence in support of the observation that inhibitors of 5-HT uptake can also act as 5-HT antagonists (Domenjoz & Theobald, 1959).

Binding of [^{3}H]-5-HT to the middle affinity site (Ka^{-1}= 0.1 μM) was insensitive to 5-HT antagonists but was blocked by 5-HT uptake inhibitors. There was good correlation between inhibition of 5-HT uptake and inhibition of binding to the middle affinity site for chlorimipramine (IC_{50} value against uptake, 0.2 μM), Lilly 103947 (0.25 μM), Lilly 110140 (0.5 μM), imipramine (0.7 μM), amitriptyline (1.2 μM) and desmethylimipramine (7.5 μM). Our results suggest that the binding of [^{3}H]-5-HT to the highest affinity site is involved in the production of the platelet shape change and that the middle affinity site may be related to the carrier for the active transport of 5-HT.

A.H D. is an M.R.C. scholar.

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A study of the binding of drugs of blood constituents

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This communication reports some preliminary results obtained in the study of the nuclear magnetic resonance spectroscopy (n.m.r) of compounds that inhibit platelet aggregation.

Platelet suspensions were prepared from citrated rabbit and human blood by a modified procedure of Ardlie & Han (1974). To aid the resuspension of platelets after centrifugation, 1 vol of a 10% sucrose solution was added to 5 vol of platelet rich plasma (PRP). Submaximal aggregation was produced by adenosine diphosphate (ADP) 2 μM and the aggregation was recorded by the turbidimetric method of Born & Cross (1963). The n.m.r. spectra were obtained using a 60 or 100 MHz spectrometer with D_2O as a solvent.

ADP-induced platelet aggregation occurred to the same degree in platelet suspensions made up in water or D_2O. This enabled the NMR spectra of compounds to be studied in washed platelet suspensions without any impairment of the ability of platelets to aggregate.

Dipyridamole, its analogues 2,6-bis(diethanol-amino)-4-piperidinopyrimido-[5,4-d] pyrimidine (RA 233), 4-morpholino-2-piperazine-thiopheno-[3,2-b] pyrimidine (VK 774) and 2-[(2-amino-ethyl)amino] -4-morpholinothiopheno- [3, 2-b] pyrimidine (VK 744) were studied together with AG19417 (CIS 1, 2, 3, 4, 4a 10b-hexahydro-8, 9-dimethoxy-2-methyl-6-phenylbenzo [c] [1,6]-naphthyridine bis hydrogen maleate) (Ott & Smith, 1971) and its 4-acetoaminophenyl analogue (AH21132).

Preliminary binding studies were performed using four or five concentrations of bovine serum albumin (BSA). Dipyridamole and its analogues gave spectra that were unsuitable for quantitative study but the addition of increasing concentrations of albumin did cause the spectral peaks of dipyridamole and its analogues to broaden.

The spectra of AG19417 exhibited single isolated peaks which allowed calculations of relaxation rates to be made. An increase in BSA concentration resulted in a linear increase in the relaxation rates of the three main peaks. The increase for the phenyl group was significantly greater ($P > 0.95$) than the increase for either the two methoxy groups or the *N*-methyl group suggesting that the phenyl group is specifically involved in the binding process.

In washed platelet suspensions (4 x 10^6 platelets/mm^3) the peaks of both AG19417 and

AH21132 gave the same relative increase in relaxation rates. The relaxation rate of the relaxation of maleate ion also increased in platelet suspensions but to a lesser degree. The greater increase in the relaxation rates of drug molecules indicates a specific binding to platelets.

The results obtained indicate that if suitable preparations of platelets can be prepared, information of binding characteristics of drugs which exhibit single peak spectra can be obtained.

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Effects of prostaglandins E_1, E_2 and D_2 on platelet aggregation: variation with animal species and ionized calcium concentration

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Prostaglandin E_1 (PGE_1) is a potent inhibitor of platelet aggregation in all animal species (Ki ∿ 20 nM). Prostaglandin D_2 (PGD_2) is even more effective than PGE_1 at inhibiting the aggregation of human platelets but is apparently much less potent when tested on platelets from some other species (Smith, Silver, Ingerman & Kocsis, 1974). Prostaglandin E_2 (PGE_2) is inhibitory at micromolar concentrations and there is some controversy as to whether lower concentrations enhance aggregation (Bruno, Taylor & Droller, 1974). These studies were performed using platelets suspended in media containing sub-physiological concentrations of ionized calcium. The effect of PGE_1 varies with the ionized calcium concentration (Vigdahl, Marquis & Tavormina, 1969) and platelet aggregation is also calcium-dependent to a variable extent in different animal species (Mürer, 1972). We have investigated the effects of PGE_1, PGE_2 and PGD_2 on collagen-induced aggregation of human, pig and rat platelets in platelet-rich plasma (PRP) anticoagulated with citrate (which chelates free calcium) or heparin (Gordon & MacIntyre, 1974, Gordon and Drummond, 1974).

Results are shown in Table 1. PGE_1 was most potent in man and least potent in the pig. PGD_2 was more potent than PGE_1 in man, less potent than PGE_1 in the pig and was inactive in the rat. PGE_2 was much less potent than PGE_1 in all species. In man and rat, all three prostaglandins were more potent inhibitors in citrate PRP than in heparinized PRP, but in the pig the reverse was true, and PGE_2 induced aggregation directly in pig heparinized PRP. PGE_2 never induced aggregation in human or rat PRP, although with concentrations around 0.3 μM, collagen-induced aggregation was enhanced in heparinized PRP but not in citrated PRP. Platelet aggregation induced by PGE_2 in pig heparinized PRP was inhibited by citrate, EDTA, PGE_1 and PGD_2.

It has been previously shown that platelet aggregation can be induced by endoperoxide intermediates in the PGE_2 biosynthetic pathway (Willis, 1974) and by synthetic derivatives of PGE_2 (Fenichel, Stokes & Alburn, 1975) but the results of the present study are the first demonstration of platelet aggregation induced by a stable, naturally-occurring prostaglandin

Table 1 Effect of prostaglandins on collagen-induced platelet aggregation IC_{50} values (μM)

Prostaglandin	*Man*		*Rat*		*Pig*	
	Citrate	*Heparin*	*Citrate*	*Heparin*	*Citrate*	*Heparin*
PGE_1	0.015	0.054	0.06	0.09	0.27	0.12
PGD_2	0.008	0.015	> 200.0	> 200.0	1.4	0.15
PGE_2	6.0	22.0	75.0	135.0	67.0	*

* Induced aggregation directly.

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Enlargement of the caecum in the rat

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Enlargement of the rat caecum has been found to occur following the repeated administration of a wide range of materials which are ingested by man. These include certain antibiotics e.g. neomycin, erythrosine (Butterworth, Gaunt, Grasso & Gangolli, 1975) and modified starches (de Groot, Til, Feron, Dreef-van der Meullen & Willems, 1974). A notable feature of the above studies was that the histological appearance of the caecal tissue was reported as normal. Thus when sub-acute and chronic toxicity studies have been performed on these materials, it has been found to be difficult to determine the biological significance of this enlargement. Experiments have been performed to determine some of the factors associated with the caecal enlargement produced by erythrosine.

Groups of 10 male, 4-week old, Wistar rats were fed a diet containing 0 or 2% erythrosine (2,4,5,7-tetraiodofluorescein) for 17 weeks. At post-mortem examination the weights of the caeca, both 'full' and empty, of these animals were 9.37 ± 1.33 g (mean and 95% interval estimate) and 1.31 ± 0.22 g respectively, compared with 4.25 ± 0.70 g and 0.90 ± 0.13 g for the control animals. The total amount of faecal material passed by the animals fed erythrosine was 137% of the control value ($P < 0.01$), although there was no comparable increase in the food or water consumptions. Examination of the caecal contents revealed statistically significant changes ($P < 0.05$) in the microflora of the treated animals when compared with the controls. The total number of coliforms was increased to 8.21 ± 0.22 g^{-1} expressed as $\log_{10}$ (controls = 6.04 ± 0.48). Similarly the strict anaerobes were increased to 10.07 ± 0.38 (control = 9.44 ± 0.17) and *Streptococcus faecalis* to 5.95 ± 0.17 (control = 5.21 ± 0.47), while the lactobacilli were reduced to 4.28 ± 1.44 (control = 8.10 ± 0.27). The staphylococcal count was reduced in the treated rats to 2.24 ± 1.30 (control = 3.68 ± 0.29), but the decrease was not statistically significant. No differences were observed in the protein, lipid, or water content when expressed per unit weight of the caecal tissue. However the DNA content was increased from 871 ± 93 to 1275 ± 333 mg 100 g^{-1} dry weight of tissue ($P < 0.05$).

It is postulated that erythrosine, in altering the bacterial flora of the caecum, reduced the breakdown of certain dietary constituents, which then were excreted in increased amounts. Although it cannot be assumed that the underlying mechanisms of caecal enlargement induced by different agents are the same, it is suggested that, in the case of erythrosine, the increase in bacteria and undigested material placed an extra load on the caecum leading to a 'work' or functional hypertrophy and possibly hyperplasia. Work is in progress to investigate this hypothesis.

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The relationship between fenfluramine and norfenfluramine blood levels and anorectic activity in the rat

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The central anorectic activity of fenfluramine is thought, in contrast to the amphetamines, to be mediated through the serotonergic neurones, although the exact locus of cerebral activity is unknown (Blundell & Lesham, 1974). There is increasing evidence to show that the de-ethylated metabolite of fenfluramine, norfenfluramine, possesses marked central (Broekkamp, Weemaes & van Rossum, 1975) and peripheral (Kirby, 1974) properties and could account for the total activity. This supposition was investigated by comparing in two experiments the combined anorectic activity of fenfluramine and norfenfluramine after fenfluramine administration and the anorectic activity of norfenfluramine alone when given at a dose which produces the same blood level as measured following fenfluramine administration.

Male, Black-Hooded rats (n = 6) were tested for food intake, using the procedure of Blundell, Campbell, Lesham & Tozer (1975) following intraperitoneal injection of ± fenfluramine HCl ($10\ \mathrm{mg\,kg^{-1}}$) or saline control (0.9 w/v NaCl). Thirty minutes after the injection, the food consumption of the animals was measured at 1, 4, 8 and 24 h after ingestion. Blood was taken from a second set of animals kept under identical conditions to the first by cardiac puncture for the measurement of fenfluramine and norfenfluramine. The peak reduction in food intake (75% over control) occurred at 1 h and remained at approximately the same intensity for approximately 24 hours. Fenfluramine blood levels peaked at 1 h (1.00 μg/ml) but rapidly declined with a half-life of 2.6 hours. Norfenfluramine levels were lower at 1 h (0.22 μg/ml) rising to a peak at 4 h (0.060 μg/ml) and then gradually declining with a half-life of 14 hours. The best fit of anorectic potency was given by the sum of the norfenfluramine and fenfluramine blood levels.

The second experiment was undertaken to investigate the contribution of norfenfluramine in the initial anorectic activity of fenfluramine. Four doses of norfenfluramine, 1, 2.0, 2.5 and 3.0 mg/kg were administered intraperitoneally to four groups of animals (n = 5). Blood samples were collected after one hour. A linear relationship was found between the dose and the 1 h blood level. The dose which produced a 1 h concentration of 0.22 μg/ml, equivalent to that which occurred after fenfluramine administration, was calculated to be between 1-2 mg/kg.

Under the same conditions as for the first experiment, it was shown that administration of norfenfluramine alone (2 mg/kg) produced a 28% reduction in food intake after one hour. This is statistically lower ($P < 0.01$) than that produced after fenfluramine administration, 75%, although both these injections gave rise to approximately the same norfenfluramine blood level

From these results, it seems likely that fenfluramine itself plays a major role in the initial anorectic potency of the drug but the prolonged activity of fenfluramine in the rat is mediated by the active metabolite, norfenfluramine.

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Effect of diazepam and a soluble salt of diazepam (fosazepam) on sleep in man

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In previous work we have studied the effect of some benzodiazepines on visuo-motor co-ordination in man, and, though with many hypnotics impaired performance persists well into the next day, it would appear that with diazepam (10 mg) the duration of impaired performance may be limited (Borland & Nicholson, 1975a & b). Diazepam, or a closely related drug, could prove to be a useful hypnotic for persons involved in skilled activity However, there has been little experimental interest in the effect of this drug on sleep, except for the brief report by Kales & Scharf (1973).

In this context we have studied the effect of diazepam and a soluble salt, fosazepam (7 - chloro - 1 - (dimethyl - phosphinylmethyl) - 5 - phenyl - 1,3 - dihydro - 2H - 1,4 - benzodiazepin - 2 - on), on sleep in man. Six healthy male volunteers, aged between 19 and 43 years, were used They were not taking drugs, and abstained from unusual exercise and the use of caffeine and alcohol from noon of each day of a sleep recording. Subjects were familiar with the laboratory, and their sleep patterns after ingestion of placebo on three separate occasions did not reveal any significant trends. The experiments were double blind. Diazepam (5 or 10 mg), fosazepam (60 or 80 mg) or placebo were ingested as identical capsules at lights out, and recordings were made on the night of administration and the next (recovery) night. Experiments were separated by at least nine days.

It was not possible to establish dose-related effects for most of the sleep measures. Diazepam (5 and 10 mg) and fosazepam (60 and 80 mg) reduced the mean sleep onset latency from 30.7 to 21.6 and 20.1 min respectively ($P = 0.01$), and with fosazepam the mean latency to stage 3 was also shortened ($P = 0.05$). Stage 0 (wakefulness) was depressed by both drugs ($P = 0.001$), and with fosazepam stage 1 (drowsiness) and stage 4 were also reduced ($P = 0.01$) Dose related effects were observed with total sleep time No change was observed after 5 mg diazepam, but with 10 mg mean total sleep time was increased from 413 1 to 457 7 min ($P = 0.05$). With fosazepam it was increased to 484 4 min after 60 mg ($P = 0.001$), and to 514 2 min after 80 mg ($P = 0.001$). On the recovery night the shortened sleep onset latency persisted with both drugs ($P = 0.01$), but there was little evidence of any other effect with diazepam, though with fosazepam there was a persistent depression of stage 1 ($P = 0.01$) and stage 4 ($P = 0.05$) sleep.

It is suggested that diazepam at 5 mg may offer some advantages as a hypnotic for occasional use by healthy persons. Sleep onset latency and awakenings are reduced without change in the overall sleep architecture or prolongation of the sleeping time. On the other hand the effects of fosazepam (60-80 mg) are more pronounced, though whether the changes in sleep patterns are dose-dependent or specific to the drug is not clear The prolonged total sleep time suggests that a lower dose range may have comparable effects with 5-10 mg diazepam.

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The relationships between liver volume and the disposition of antipyrine and indocyanine green before and after phenobarbitone treatment

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A relationship has previously been reported in normal subjects and in patients with chronic liver disease between the clearances of antipyrine and indocyanine green (Branch, James & Read, 1975). Antipyrine is a drug which is slowly metabolized by the hepatic microsomes and its clearance is independent of liver blood flow. Indocyanine green is actively and rapidly excreted into bile and its clearance is therefore limited by liver blood flow. It has been suggested that the common rate-limiting factor is the total functional hepatic mass. Phenobarbitone increases the concentration of mixed function oxidase enzymes in man (Lecamwasam, Franklin & Turner, 1975) and increases liver weight and blood flow in the rat (Ohnhaus, Thorgiersson, Davies & Breckenridge, 1971) and in the rhesus monkey (Branch, Shand, Wilkinson & Nies, 1974).

The effect of oral administration of 180 mg/day of phenobarbitone for three weeks on liver volume and clearances of antipyrine and indocyanine green has been investigated in ten normal subjects. Liver volumes were determined by an ultrasonic scanning technique similar to that of Rasmussen (1972). Antipyrine was assayed by the method of Brodie, Axelrod, Soberman & Levy (1949) and indocyanine green by the method of Caesar, Shaldon, Chiandussi, Guevara & Sherlock (1961). Dosages and sampling procedures were as described previously (Branch, *et al.*, 1975).

The technique used for liver volume estimation was reproducible. One subject had a very high initial antipyrine clearance and this did not change after phenobarbitone administration indicating that this subject was maximally induced at the start of the study. The antipyrine clearance of the other nine subjects correlated with liver volume ($r + 0.69$, $P < 0.05$). The initial clearances of indocyanine green of seven subjects approximated to the expected liver blood flow and related to liver volume. The indocyanine green clearance in the other three subjects was lower than predicted by this relationship.

After phenobarbitone there was no increase in liver volume. The clearance of antipyrine increased by 90 ± 14.1% ($P < 0.001$) (mean ± s.e mean). There was still a correlation between antipyrine clearance and liver volume ($r + 0.86$, $P < 0.01$) There was no significant increase in indocyanine green clearance. The relationship between the indocyanine green clearance and liver volume was maintained in the same seven subjects who had demonstrated such a relationship prior to phenobarbitone. The clearances in the other three subjects remained low.

In conclusion, the effect of phenobarbitone on the interrelationships examined suggests that, in the absence of enzyme induction, variation in drug elimination is related to the total functional hepatic mass, and that phenobarbitone is able to increase the drug metabolizing capacity per unit of mass, without causing an increase in total liver size.

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A correlation between the response to debrisoquine and the amount of unchanged drug excreted in the urine

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The correlation between responsiveness to the adrenergic blocking agent debrisoquine and the amount of unchanged drug excreted in the urine has been investigated in normotensive subjects and in a number of hypertensive outpatients as part of a study on the therapeutic implications of metabolism of this hypotensive agent.

Preliminary experiments showed that single oral doses (40 mg) of [^{14}C]-debrisoquine were well absorbed (75% of ^{14}C was excreted in the urine in 24 h) and that the drug was metabolized by hydroxylation to an extent which appeared to vary between subjects (Angelo, Dring, Lancaster & Smith, unpublished data). To investigate a possible relationship between responsiveness and extent of metabolism in a larger group of subjects a g.l.c. method for the estimation of unchanged debrisoquine in urine was developed. This was a modification of the method of Hengstmann, Falkner, Watson & Oates (1974) and depends upon the hydrolytic conversion of debrisoquine to tetrahydroisoquinoline which is measured as its trichloroacetyl derivative on g.l.c. with electron capture detection using phenformin as internal standard.

For the purpose of this study the blood pressure of 10 normotensive subjects with normal renal function who had received 40 mg of debrisoquine orally was recorded lying down, standing and after walking 50 yards, using a random zero sphygmomanometer, at intervals for up to 48 hours. Urine samples were collected at similar intervals for measurement of their debrisoquine content. The blood pressure of the subjects was measured on a control day at the same times but in the absence of the drug.

There occurred a sevenfold range (8-58% of dose) in the amount of debrisoquine excreted in the urine unchanged. The mean of the standing blood pressure values obtained 2, 3, 4, 6, 8, 10 and 24 h after debrisoquine was compared to the values obtained at identical times on a control day and showed that the three subjects with a statistically significant ($P < 0.05$) hypotensive response excreted more unchanged debrisoquine (34, 51, 58% of the dose) than seven non-responders ($P < 0.005$) who excreted a mean of only 16% of the dose unchanged (range 8-28). The subject who excreted most unchanged drug had a standing mean blood pressure more than 2 s.d. below his control value 32 h after ingestion.

A number of hypertensive outpatients with normal renal function were treated with debrisoquine and the hypotensive response determined by comparing their pre-debrisoquine mean standing blood pressure with that found after the drug. The amount of unchanged debrisoquine excreted in the urine in the 24 h following drug administration was also determined. The range of percentage of the dose excreted unchanged in the urine (8-48%) was similar to that found in normotensive subjects and the correlation between response to the drug and the amount of unmetabolized drug excreted was better than between the response and the daily dose.

It is concluded that probably debrisoquine itself and not a metabolite is responsible for its hypotensive action and that the rate at which debrisoquine is metabolized is a major determinant of responsiveness to the drug. Inter-individual difference in response to the drug can be related to variations in the extent of metabolism and not to differences in absorption.

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A light and electron microscopic study of the accumulation of material in sectioned rat dorsal roots and the effect of demecolcine

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Accumulation of vesicular material has been demonstrated in sectioned, crushed and ligated nerves *in vivo* and *in vitro* (Dahlstrom, 1971). The following experiments were carried out in order to examine the effects of sectioning rat dorsal roots as a basis for the study of the nature, formation and transport of axoplasmic material in sensory neurones.

Rats (250 g) were anaesthetized with urethane and dorsal roots L3-5 were sectioned. Twenty hours later the animals were perfused with glutaraldehyde (2.5%) and the sectioned roots were examined by light and electron microscopy.

The nerve on the ganglionic side of the cut showed numerous unmyelinated profiles at the cut tip, many of them packed with vesicles. It was seen from longitudinal sections that some of these unmyelinated 'sprouts' arose from myelinated axons. In both myelinated and non-myelinated axons there was an accumulation of mitochondria and vesicles (60-100 nm in diam., some dense-core) for a distance of 500 μm behind the tip. With increasing distance from the tip, the proportion of mitochondria increased and the accumulation became confined to the periphery of the axoplasm. The nerve on the side of the cut distant from the ganglion showed no unmyelinated profiles at the cut end. In addition, the myelin was degenerate, the Schwann cells were active, and the axoplasm of both myelinated and non-myelinated axons was packed with debris including some vesicles and many lamellated bodies.

When dorsal root ganglia with a length of root attached were cultured *in vitro* by the method of Trowell (1959) for 20 h in Medium 199 (Flow Laboratories), the appearance of the cut-end of the nerve was similar to that of a nerve sectioned *in vivo*. However, when an equivalent segment of dorsal root without the ganglion attached was cultured for 20 h the appearance of the axoplasm some distance from the cut end was normal but there were no unmyelinated profiles at the tip, nor was there any accumulation of mitochondria or vesicles.

When intact ganglion-nerve preparations were cultured for 20 h in the presence of demecolcine (3×10^{-7} M) no axonal sprouting or accumulation of mitochondria and vesicles was seen.

In conclusion it appears that in sectioned dorsal roots axonal sprouting and accumulation of material occur only in the presence of cell bodies. Demecolcine (3×10^{-7} M), which disrupts neurotubules, prevents this accumulation and sprouting. Further work on this preparation is necessary to determine the role of axonal transport in these processes and their importance in neuronal function.

This work was supported by a grant from the Medical Research Council.

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Proconvulsant action of folic acid

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Folic acid (FA) is convulsant when injected intracerebroventricularly (i.c.v.) in mice (Baxter, Miller & Webster, 1973). It has been suggested that in epilepsy high localized folate concentrations could form epileptic foci (Hommes & Obbens, 1972). Microiontophoretic FA has been shown to either stimulate cortical cells or to enhance the effect of applied glutamate (Hill, Miller, Straughan & Webster, 1973). As increased FA may therefore predispose to convulsions we have studied its proconvulsant action (i.c.v.) in mice and rats and compared it with other convulsants by the same route.

In threshold electroshock studies using mice, the normal clonic response was intensified to hind limb extension (HLE) by FA pre-treatment. The ED_{50} values for HLE for FA alone and with electroshock 15 min later were 20.5 and 1.5 μg respectively (ratio = 13.7). Picrotoxin and ouabain also had strong proconvulsant actions (ratios 97 and 91 respectively), maximal at 15 min like FA. Ouabain had a longer duration of action (at least 4 h) than FA and picrotoxin (1 h) Other convulsants tested (glutamate, homocysteic acid, leptazol and strychnine) were only weakly active immediately (5-60 s) after administration and had low ratios (1.4 to 2.7). In the leptazol infusion test (0.3 mg/min i v.) in mice all drugs tested except ouabain when administered at up to convulsant ED_{50} values significantly reduced the time from clonus to HLE ($P < 0.05$)

The proconvulsant action of sub-convulsive doses of FA in mice to a strong afferent stimulus which was itself not convulsant was examined by subjecting the mice to an auditory stimulus (110dB, 15 s) from a bell suspended at 70 cm in a box 34 cm diameter and 76 cm high. In mice pre-treated (−15 min) with FA, generalized convulsions (ED_{50} = 2.3 µg) and HLE (2.8 µg) were readily induced. Convulsions occurred in only 4% of mice ($n = 90$) pretreated with control fluid (i c.v.).

It is known that repetitive electrical stimulation applied locally to the cortex can act as an epileptogenic focus which will produce an after-discharge (AD). ADs were induced in rats chronically implanted with an electrical connector linked to screw electrodes in the skull (Goff, Miller, Smith, Smith & Wheatley, 1975). Stimulation (100 Hz, 1 ms width, 1 s duration) through a frontal screw induced clonic convulsions accompanied by AD of mean duration 12.1 s ($n = 9$). When tested 90 min later, FA (10 µg intraventricularly) at 15 min before the second stimulation significantly increased AD duration by 90% ($P = 0.001$ $n = 3$) Control fluid (intraventricularly) significantly reduced AD duration by 62% ($P = 0.05$. $n = 3$) whereas in untreated rats ($n = 8$), AD duration was unchanged after the second stimulation.

The results suggest that raised folate concentrations can increase cortical activity and therefore increase the tendency to convulsions.

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Further observations on the change in sensitivity to halothane induced by acute administration of central nervous system depressant drugs in the rat

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In a previous communication we outlined the use of repeated determination of halothane-induced sleeping time as a method for studying changes in the excitability of the central nervous system (CNS) occurring with time (Turnbull & Watkins, 1975). We reported that pre-treatment of rats with sodium pentobarbitone (a total dose of 90 mg kg^{-1} over a period of 10 h) or meprobamate (800 mg kg^{-1} 10 h^{-1}) resulted in the development of a hyposensitivity to halothane which was followed by a rebound hypersensitivity to the anaesthetic. However, we could not be certain that the changes in sleeping time were entirely due to changes in the sensitivity of the CNS. We have therefore measured brain halothane concentrations on awakening in saline and drug pretreated rats and have found the same pattern of change in CNS excitability as was indicated by the sleeping time.

First, we have confirmed that repeated exposure to halothane does not induce hyposensitivity. The brain halothane concentration found on awakening from the last of twelve exposures to halothane was the same (111 ± 10 (6) µg g^{-1} ± s.e. mean) as that found in rats which had been anaesthetized only twice during the same 48 h period (120 ± 6 (6) µg g^{-1}). Secondly, we have shown that the diurnal variation in sleeping time is due to an altered sensitivity of the CNS to halothane, since rats awakened with a higher brain halothane content at 0100 h (167 ± 7 (6) µg g^{-1}) than at 1100 h (126 ± 11 (6) µg g^{-1}).

We have also repeated our experiments in which halothane sleeping time was determined at intervals after administration of sodium pentobarbitone (90 mg kg^{-1} 10 h^{-1}) or meprobamate (800 mg kg^{-1} 10 h^{-1}) but in addition have determined the brain halothane concentration on awakening in groups of similarly pretreated animals killed at the time of maximum hyposensitivity and hypersensitivity to the anaesthetic The brain halothane concentration on awakening was significantly higher at the time of decreased sensitivity (control 131 ± 5: pentobarbitone pretreated 157 ± 8; meprobamate pretreated 177 ± 9 $\mu g\, g^{-1}$) and significantly lower at the time of hypersensitivity (control 190 ± 23: pentobarbitone pretreated 125 ± 21, meprobamate pretreated 132 ± 18 $\mu g\, g^{-1}$) compared with the levels found in saline pretreated animals.

In addition, we have assessed the sensitivity of similarly pretreated rats to pentobarbitone by determining the duration of anaesthesia following an i.c.v. injection of 800 µg sodium pentobarbitone. Halothane-tolerant rats were found to be tolerant to i.c.v. pentobarbitone and halothane-sensitive rats slept for significantly longer than control animals. Thus, using three different indices, our results indicate that repeated injection of anaesthetic doses of pentobarbitone or meprobamate leads to the development of a hyperexcitability which is followed by a rebound decrease in the excitability of the CNS. The effect of pretreatment with other centrally active drugs is presently being investigated.

We are grateful to the Medical Research Council for financial assistance

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Is morphine inhibition of the twitch response of the mouse vas deferens mediated via noradrenaline?

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The inhibitory effect of morphine on the mouse vas deferens was first reported by Henderson, Hughes & Kosterlitz (1972). We have confirmed that morphine in low concentrations (0.03-3.0 µM) inhibits the twitch response to field stimulation (0.1 or 1.0 Hz, 1 ms, 150 mA). This action of morphine is antagonized by small doses of the narcotic antagonist, naloxone (50 nM).

Morphine has also been reported to inhibit the output of noradrenaline in this tissue and it was suggested that this was the mechanism by which it inhibited the twitch response (Henderson, Hughes & Kosterlitz, 1972; Hughes, Kosterlitz & Leslie, 1975).

In 5 experiments the output of noradrenaline from 4 vasa deferentia was measured by bioassay (Hughes, 1972). After 120 stimuli at 1.0 Hz it was 52 ± 9 pg (mean ± s.e. mean). When morphine (1.0 µM) was added to the bath in the same experiments the output of noradrenaline was 53 ± 9 pg. After the morphine was washed out 43 ± 13 pg of noradrenaline was released.

In another experiment a control output of 31 pg of noradrenaline was increased by phenoxybenzamine (15 µM) to 391 pg. Here morphine (1.0 µM) still inhibited the response to stimulation but did not reduce the output of noradrenaline (373 pg).

When noradrenaline (0.1-3.0 µM) was added to the bath the twitch was inhibited and this inhibition was reduced by phentolamine (10 µM). The dose response curve for the inhibitory effect produced by morphine (0 1-0.3 µM) was unaffected by phentolamine (10 µM). Conversely, the dose-response curve for the inhibitory effect of noradrenaline was unaffected by naloxone (50 nM) while the same concentration of naloxone displaced the morphine curve to the right.

The motor response of the vas deferens to exogenous noradrenaline or acetylcholine was unaffected by morphine (1.0 µM) thereby excluding an action of the drug on post-synaptic receptors.

In conclusion, these results suggest that the inhibitory effect of morphine on the twitch response of the mouse vas deferens is unlikely to be mediated via noradrenaline.

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The effects of chronic lithium administration on the metabolism of L-tryptophan in the rat forebrain

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We have investigated the effect of lithium (Li^+) on the accelerated production of 5-hydroxyindoles induced by a large dose of L-tryptophan. Male Albino Wistar rats (124) weighing 150-250 g were divided into two groups of 62 animals. One group received an i.p. injection of isotonic (0.15 M) LiCl (0.75 mequiv/kg) each day for 10 days, whilst the control group received injections of saline. Twenty-four hours after the last dose of Li^+ or saline, half of the animals in each group received a single injection of L-tryptophan (100 mg/kg i.p.) whilst others received an equivalent volume of saline. Pairs of animals (one tryptophan treated and one control) from each group were killed 30, 60 and 90 min after the injection of tryptophan or saline, and the forebrain concentration of 5-HT and 5-HIAA measured fluorimetrically (Collard & Roberts, 1974).

Li^+ pretreatment had no effect on the changes in forebrain 5-HT and 5-HIAA measured 30 or 90 min after the injection of L-tryptophan (Table 1). However, the maximum increase in 5-HT concentration which occurred 60 min after the injection, was significantly reduced in the Li^+ group, and the concentration of 5-HIAA was correspondingly increased.

There is evidence that the increase in 5-HIAA concentration induced by a loading dose of L-tryptophan results from the deamination of the increased amount of free cytoplasmic 5-HT which is accessible to monoamine oxidase (Moir & Eccleston, 1968). The results of this study may therefore indicate that Li^+ increases the deamination of free cytoplasmic 5-HT, possibly by inhibiting the transport of newly synthesized 5-HT into, or the binding of 5-HT within the storage compartment of 5-HT neurones Alternatively, Li^+ may divert the metabolism of L-tryptophan away from the production of 5-HT, and towards the production of 5-hydroxyindolepyruvic acid through the 5-HTP aminotransferase pathway (Millard & Gal, 1971).

Table 1 The effect of Li^+ pretreatment on L-tryptophan-induced changes in forebrain 5-hydroxyindoles measured 30, 60, and 90 min after the i.p. injection of L-tryptophan (100 mg/kg). Results are expressed as the mean ± s.e. of mean 8, 13, and 10 pairs respectively. Statistical analysis is by paired *t* test

	Change in 5-HT concentration (ng/g wet weight)			
Time after L-tryptophan	*Control*	*Lithium*	*Lithium minus control*	*P*
30 min	+ 145 ± 50	+ 142 ± 52	−3 ± 77	NS
60 min	+ 283 ± 59	+ 132 ± 71	−151 ± 64	$P < 0.05$
90 min	+ 202 ± 53	+ 72 ± 80	−130 ± 96	NS

	Change in 5-HIAA concentration (ng/g wet weight)			
Time after L-tryptophan	*Control*	*Lithium*	*Lithium minus control*	*P*
30 min	+ 75 ± 19	+ 117 ± 15	+ 42 ± 24	NS
60 min	+ 201 ± 39	+ 280 ± 23	+ 79 ± 35	$P < 0.05$
90 min	+ 254 ± 30	+ 235 ± 47	− 19 ± 39	NS

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Influence of etorphine acepromazine and diprenorphine on respiratory function in ponies

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Although a neuroleptanalgesic drug combination (Large Animal Immobilon: Reckitt & Colman Ltd.) containing etorphine and acepromazine has been used in veterinary medicine for several years, its effects on respiratory function have not been reported in detail. In this study the drug combination was administered i.v. (25 μg/kg etorphine and 100 μg/kg acepromazine) to twelve Welsh Mountain ponies ranging from 185 to 336 kg bodyweight. Arterial blood samples were collected for analysis before and at fixed times after the injection. An antagonist to etorphine, diprenorphine (Revivon; Reckitt and Colman Ltd.), was then administered i.v. at a dose level of 30 μg/kg and further blood samples were obtained.

Respiration was depressed throughout the period of neuroleptanalgesia with the ponies lying in lateral recumbency, as indicated by pronounced reductions in respiratory rate and arterial oxygen tension ($P\text{O}_2$), although arterial carbon dioxide tension ($P\text{CO}_2$) was increased only slightly (Table 1). The decrease in $P\text{CO}_2$) was attributed to the etorphine-induced reduction in respiratory rate, the position of lateral recumbency which produces ventilation/perfusion abnormalities in large species, and the laboured breathing pattern which accompanied the etorphine-induced increase in voluntary muscle tone. In spite of the pronounced hypoxic hypoxia, arterial oxygen content (CO_2) was not reduced significantly and arterial oxygen flux (FO_2) actually increased as a result of compensatory rises in Hb concentration and cardiac output.

Table 1 Effects of etorphine, acepromazine and diprenorphine on respiratory function

Time (min)	*$P\text{O}_2$ (mmHg)*	*$P\text{CO}_2$ (mmHg)*	*CO_2 (ml/100 ml)*	*FO_2 (l/min)*
Control	96 ± 1	44 ± 1	16.1 ± 0.4	2.51 ± 0.25
E + 5	50 ± 2**	51 ± 2**	15.9 ± 0.6	3.64 ± 0.34*
E + 15	51 ± 2**	52 ± 2**	14.9 ± 0.4	3.01 ± 0.20*
E + 30	53 ± 3**	50 ± 2**	14.7 ± 0.6	3.19 ± 0.32**
D + 5	83 ± 4**	45 ± 2	16.0 ± 0.5	3.16 ± 0.49
D + 15	88 ± 3*	46 ± 1	14.5 ± 0.3**	2.54 ± 0.27
D + 30	86 ± 3*	47 ± 1*	13.3 ± 0.3**	2.10 ± 0.27
D + 60	85 ± 3**	46 ± 1	12.7 ± 0.3**	1.89 ± 0.16**

Values are means with s.e. mean for twelve ponies. Times refer to the i.v. administration of etorphine and acepromazine (E) or the i.v. injection of diprenorphine (D). The significance of differences from control values was assessed by paired *t*-tests and is indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$.

Following diprenorphine injection, $P\text{CO}_2$ and $P\text{O}_2$ returned to near normal levels but CO_2 and FO_2 fell progressively as a result of a gradual reduction in Hb concentration.

This study was supported by the Wellcome Foundation and the Horserace Betting Levy Board. Drugs were supplied by Reckitt and Colman Ltd.

Responses of the isolated sphincter of Oddi from the guinea-pig to field stimulation

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Recent reviews (Persson, 1972; Sarles, 1974; Tansy, Innes, Martin & Kendall, 1974) have highlighted the uncertainties concerning the control of the terminal portion of the common bile duct. The present study was undertaken to investigate the neurally mediated responses of the guinea-pig sphincter of Oddi to field stimulation and their modification by drugs.

The choledochoduodenal junction of the guinea-pig is anatomically noteworthy. The lower end of the common bile duct expands into a large oval pouch lying on the serosal surface of the duodenum. From the caudal end of the pouch a small duct passes directly into the duodenum (Higgins, 1927).

Male guinea-pigs (200 to 1,000 g) were stunned, bled and the terminal portion of the common bile duct and surrounding duodenal areas were removed. A polyethylene cannula (1 mm i.d.) was passed, via the common bile duct, into the pouch and ligated. The duodenum was trimmed to the limits of the pouch. The cannula was attached to a Y-tube one arm of which was connected to a pressure transducer linked to a polygraph, the other was perfused with Krebs solution at the rate of 0.024 ml/min. The preparation was then placed in a bath of Krebs solution maintained at 37° C and bubbled with 5% CO_2 in oxygen. Field stimulation was applied via two platinum loop electrodes (2 cm apart at 50 V, 40 Hz and 0.2 ms pulse width.

The preparations exhibited resting perfusion pressures of 1 to 6 cm H_2O with approximately one third showing spontaneous activity. Field stimulation resulted in changes in perfusion pressure of up to 25 cm H_2O. The increase in resistance to perfusion with field stimulation was abolished by tetrodotoxin (1 μg/ml) and lignocaine (117 μg/ml). This stimulation response was also much reduced in 30 of 44 preparations by atropine (28 ng/ml) or hyoscine (152 ng/ml). Acetylcholine (0.05 to 50 μg/ml) and carbachol (1 to 5 μg/ml) produced increases in perfusion pressure whilst neostigmine (10 to 100 μg/ml) caused an increase in spontaneous activity and a sustained increase in perfusion pressure. Propranolol (5 μg/ml), phentolamine (140 ng/ml) or guanethidine (1.9 μg/ml) did not affect the response to field stimulation. Noradrenaline (0.5 to 2 μg/ml) in a single instance increased perfusion pressure. Isoprenaline (0.5 to 2 μg/ml) consistently inhibited activity in spontaneously active preparations. Nicotine (0.5 to 16 μg/ml) caused a small rise in perfusion pressure in 3 out of 11 preparations. This response was blocked by hexamethonium (10 μg/ml).

In this preparation, therefore, there is evidence of cholinergic neurones having a motor function, but little evidence for significant adrenergic motor control.

S Brooks is in receipt of a grant from the M.R.C.

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Peptide hormones and the extinction of conditioned taste aversion

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When a rat is subjected to the unpleasant effects of a drug contingent upon drinking a distinctively flavoured novel solution, it acquires a strong aversion to the taste of that solution (Rozin & Kalat, 1971). This phenomenon is commonly called 'conditioned taste aversion'.

Rats, which were deprived of water for 23.30 h, were allowed to drink a 5% glucose solution ('sugar water') in a single 15 min session. Lithium chloride (LiCl, 0.15 M 10 ml/kg i.p.) injected 30, 60 or 120 min after the conclusion of the drinking session induced strong avoidance of the sugar water at subsequent sessions. Avoidance was less as the interval between drinking and drug treatment was increased. Conditioned taste aversion only occurs when the test solution is novel. It was found that LiCl, given 30 min after the drinking session, could not induce avoidance for tap water or sugar water if the rats had previously been given either of these solutions in the absence of LiCl treatment

De Wied and coworkers (de Wied, 1969, Garrud, Gray & de Wied, 1974) have shown that ACTH-analogues modulate the extinction of shock or appetite motivated behaviour. It was therefore deemed of interest to study the effects of these analogues on the extinction of conditioned taste aversion. In each experiment eight groups of ten rats were used Two groups received extinction sessions (a single 15 min session/day) during which time the rats were allowed free access to sugar water contained in a drinking tube These animals had the choice between drinking the aversive solution or not drinking at all ('forced extinction'). Two other groups had the choice of either tap water or sugar water (preference test). Extinction in the preference test took much longer than forced extinction (approximately 3 weeks *versus* 3 days). In addition, two control groups which had been treated with saline instead of LiCl, were subjected to the forced extinction test and two other control groups to the preference test. In each block of two groups, one group was injected s.c. with a drug 1 h prior to daily extinction sessions whereas the other group was treated with placebo. In the first experiment the effects of ACTH (adrenocorticotrophic hormone)-analogue, ACTH$_{4\text{-}10}$, were studied. ACTH$_{4\text{-}10}$ is known to delay the extinction of shock and appetite motivated behaviour (de Wied, 1969, Garrud, Gray & de Wied, 1974). In the conditioned taste aversion test, it was found that 100 µg ACTH$_{4\text{-}10}$/rat delayed the extinction in the preference test, but did not affect forced extinction. The peptide did not influence the fluid intake of the control groups.

When the phenylalanine residue in position seven of the sequence ACTH$_{4\text{-}10}$ is replaced by its D-isomer (ACTH$_{4\text{-}10\,7\,\mathrm{DPhe}}$) extinction of shock and appetite motivated behaviour is facilitated (de Wied, 1969; Garrud, Gray & de Wied, 1974). However, ACTH$_{4\text{-}10}$ and ACTH$_{4\text{-}10\,7\mathrm{D\,Phe}}$ have a similar effect on passive avoidance behaviour· both compounds potentiate weak passive avoidance responses (Greven & de Wied, 1973). It appeared that ACTH$_{4\text{-}10\,7\mathrm{D\,Phe}}$ (100 µg/rat) delayed the extinction of conditioned taste aversion in the preference test. The peptide did not alter forced extinction or fluid intake of the control groups. The finding that ACTH$_{4\text{-}10}$ and ACTH$_{4\text{-}10\,7\mathrm{D\,Phe}}$ had similar effects suggests that extinction of conditioned taste aversion is not completely comparable to extinction of shock motivated (active) avoidance responses.

α-MSH (melanocyte stimulating hormone) and ACTH share the amino acid sequence 4-10. Like ACTH, α-MSH delays the extinction of shock and appetite motivated behaviour (de Wied, 1969, Kastin, Dempsey, LeBlanc, Dyster-Aas & Schally, 1974). It was shown that 100 µg/rat α-MSH similarly delayed the extinction of conditioned taste aversion in the preference test but not in the forced extinction test In contrast, melanocyte stimulating hormone release inhibiting factor (MIF; 100 µg/rat), which according to Sandman, Alexander & Kastin (1973) exerts behavioural effects similar to α-MSH, was without effect.

It is concluded that extinction of conditioned taste aversion can be modulated by the administration of peptide hormones or hormonal analogues

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Corticotrophin production by rat adenohypophysial segments *in vitro*

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A method for the direct assessment of the adrenocorticotrophic activity of pituitary segments has been developed using the cytochemical bioassay for corticotrophin (ACTH) (Chayen, Loveridge & Daly, 1972; Alaghband-Zadeh, Daly, Bitensky & Chayen, 1974). The responsiveness of the rat adenohypophysis *in vitro* to some substances known to affect adrenocorticotrophic activity has been studied. Anterior pituitary glands were rapidly removed from freshly killed male albino Sprague-Dawley rats, carefully bisected and incubated for 2.5 h at 37°C in an artificial medium (Bradbury, Burden, Hillhouse & Jones, 1974) gassed with 95% O_2/5% CO_2. The medium was then replaced with similar medium containing the test substance Fifteen minutes later the ACTH contents of the medium and the adenohypophysial tissue were determined separately. Lysine vasopressin, arginine vasopressin and acid hypothalamic extracts (Hiroshige, Kunita, Yoshimura & Itoh, 1968) resulted in marked, dose-related increases in both pituitary ACTH release and content. Cortisol (10-500 ng/ml) added to the final incubation medium inhibited the release but not the synthesis of ACTH induced by these substances. The method is potentially useful both for the identification and the assay of corticotrophin releasing hormone (CRH).

We are grateful to the Medical Research Council for a generous grant for the purchase of equipment.

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The effects of glucagon on the hepatic arterial vasculature of the dog: an inhibition of the effects of vasoconstrictor agents

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Glucagon is released from the pancreas and small intestine, and its effects on the hepatic vasculature may well be of functional significance. In these experiments, the action of glucagon, and its effects on responses of the hepatic arterial vasculature to noradrenaline, angiotensin and vasopressin have been examined.

Seven female dogs (10-16 kg) were anaesthetized with chloralose (Kuhlman, Paris; 50 mg kg^{-1}) and urethane (BDH; 500 mg kg^{-1}) i.v., after induction with methohexitone sodium ('Brietal', Lilly; 7.5-10.0 mg kg^{-1}, i.v.). The hepatic artery, dissected free from its periarterial sympathetic nerves which were then divided, was cannulated close to its origin from the aorta, and perfused with blood from a cannulated femoral artery. The hepatic arterial perfusion pressure was measured close to the cannula in the hepatic artery, and blood flow in this system measured with a cannulated flow probe and electromagnetic flowmeter.

The initial control values (means ± s.e. means) were hepatic arterial mean perfusion pressure (HAPP), 119.2±5.9 mmHg and hepatic arterial blood flow (HABF) 38.8 ± 5.3 ml'min^{-1} 100 g^{-1}. The calculated hepatic arterial vascular resistance (HAVR) was 3.31 ± 0.38 mmHg ml^{-1} min 100 g. *Post-mortem*, the livers weighed 324.8 ± 69.6 (mean ± s.d.) grams.

In 5 experiments, noradrenaline acid tartrate ('Levophed', Winthrop; 10 μg base, i.a.) caused a significant ($P < 0.001$; paired *t*-test) increase in hepatic arterial vascular resistance (HAVR) of 129.8 ± 19.7% ($n = 11$ injections). Angiotensin amide ('Hypertensin', Ciba; 0.5 μg salt, i.a.) caused a significant ($P < 0.001$) rise in HAVR of 161.1 ± 25.5% ($n = 12$), and vasopressin ('Pitressin', Parke-Davis; 0.1 unit, i.a.) caused a significant rise ($P < 0.01$) in HAVR of 98.7 ± 22.4% ($n = 9$).

Glucagon hydrochloride (Lilly, 100 μg base, i.a.) was injected on 14 occasions to 5 preparations, causing a transient but significant ($P < 0.001$) reduction in HAVR of 19.9 ± 3.3%, an effect similar to that found by Bashour, Geumei, Nafrawi & Downey (1973). One minute after these glucagon injections, the increase in HAVR in response to noradrenaline (10 μg, i.a.) was 14.9 ± 4.2% of the control effect to angiotensin (0.5 μg, i.a.) 5.1 ± 2.4%, and to vasopressin (0.1 *u*, i.a.) 11.0 ± 4.5% showing highly significant ($P < 0.001$) attenuation of the vasoconstrictor effects of these compounds. The vasoconstrictor responses progressively returned to control values within 8-10 min after the glucagon injections.

When glucagon was infused into the hepatic artery in dose from 10-50 μg min^{-1} for 11-13 min, the vasoconstrictor effects of noradrenaline, angiotensin and vasopressin were suppressed throughout the course of the infusions.

Glucagon has been shown to reduce the effects of α-adrenoceptor stimulation on the superior mesenteric arterial vascular bed (Kock, Tibblin & Schenk, 1971); the present study has shown that a similar attenuation of the effects of vasoconstrictor agents on the hepatic arterial bed is not limited to α-adrenoceptor agonists. This action of glucagon represents a protection of the hepatic arterial vasculature from the effects of circulating vasoconstrictor agents in states by hypoglycaemia when mobilization of hepatic glycogen would be necessary.

This study was supported by the Medical Research Council; we thank Miss June Pyke and Miss Dorinda Lobendhan for technical assistance.

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Investigations concerning TRH-induced hypothermia in cats

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Thyrotrophin releasing hormone (TRH) has been known to exert an essential function in normal thyroid metabolism for some years but recently it has been investigated in a wider context. In 1974 Metcalf reported that TRH produced hypothermia when injected into the cerebral ventricles of the unanaesthetized cat but hyperthermia when injected similarly into the rabbit. The present experiments were undertaken to compare the hypothermic effect of TRH with that of noradrenaline (NA) and calcium ions (Ca^{++}) both of which cause hypothermia in the cat (Feldberg & Myers, 1963; Myers & Veale, 1971). In addition experiments were undertaken with the amino acids which compose TRH and the thyroid hormones which it releases to ascertain whether the hypothermic action of TRH was related to its endocrine functions.

TRH (50-200 ng), NA (25-200 μg), and Ca^{++} (20-80 mM in excess of the amount found in CSF) all produced dose-related falls in body temperature when injected intraventricularly (i.c.v.) into the unanaesthetized cat. Of the three TRH was the most potent and it was estimated that the equi-potent molar ratio TRH; NA; Ca^{++}; was 1; 900; 27,000. Unlike NA or Ca^{++} the doses of TRH necessary to produce hypothermia compared well with the estimated endogenous concentration of TRH (Winokur & Utiger, 1974). In addition to hypothermia TRH induced profuse salivation, tachypnoea, cutaneous vasodilatation and frequent defecation and vomiting. Animals given NA or Ca^{++} rarely exhibited these symptoms and usually appeared quiet or sedated. Statistical analysis demonstrated a positive correlation ($r = +0.82$; $P < 0.001$) between the increased respiratory rate and the fall in temperature observed after TRH injections. Thus, the heat loss caused by panting is considered an important factor in TRH induced hypothermia in the cat but does not explain other central actions reported for TRH.

Prior treatment of cats with α-methyltyrosine (100 mg i.c.v.) depletes cerebral stores of noradrenaline (Cranston, Hellon, Luff & Rawlins, 1972) and prevents the hypothermia induced by tyramine which acts indirectly via noradrenaline release (Metcalf & Myers, 1975). Pretreatment with α-methyltyrosine did not prevent hypothermia induced by either TRH or Ca^{++}. Similarly the α-receptor antagonist, phentolamine (125 μg i.c.v.), did not affect TRH or Ca^{++}-induced hypothermia but antagonized hypothermia provoked by NA. Although TRH is a tripeptide composed of pyroglutamic acid, histidine and prolineamide, none of these amino acids (0.1 μg, i.c.v.) produced effects similar to TRH. Thyroxine (0.25-1.0 μg, i.c.v.) caused a dose-related rise in temperature of slow onset (2 h) consistent with its known effect as a metabolic stimulant whereas thyrotropin (TSH) (0.2-0.8 i u., i.c.v.) produced a gradual, sustained hypothermia which was unrelated to dose and which was not accompanied by an increase in respiratory rate.

It is concluded that the hypothermia produced in the cat by the i.c.v. injection of TRH is unrelated to its endocrine function and is not mediated by cerebral noradrenaline.

G.M. acknowledges the receipt of a travel grant from the Wellcome Trust.

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Central hypertensive action of histamine in rats

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Intraventricular (i.c.v.) injection of histamine in cats has been shown to produce a short lasting rise in blood pressure, with an associated tachycardia (Trendelenburg, 1957; White, 1965; Sinha, Gupta & Bhargava, 1969). Similarly Brezenoff & Jenden (1969) were able to show a pressor response to intrahypothalamic injection of histamine in rats. However, Corrêa & Graeff (1969) and Jagiello-Wojtowicz (1973) failed to demonstrate the hypertensive response to i.c.v. histamine in rats.

In this study we have shown that i.c.v. administration of histamine elicits increases in blood pressure and heart rate in anaesthetized rats and we have examined possible mechanisms of action involved in these histamine-mediated cardiovascular changes.

Histamine (0.1-10 μg i.c.v.) caused a dose-related increase in blood pressure and heart rate in urethane-anaesthetized rats. Pretreatment with mepyramine (50 μg and 100 μg i.c.v.) produced a dose-related antagonism of the cardiovascular effects induced by a sub-maximal dose of histamine (1.0 μg i.c.v.). However pretreatment with metiamide, a histamine-H_2 receptor antagonist, in doses of 100 and 200 μg i.c.v., or procaine (300 μg i.c.v.) did not significantly modify the cardiovascular effects of histamine (1.0 μg i.c.v.) ($P > 0.05$).

Intraventricular administration of phentolamine (100 and 200 μg i.c.v.) caused a dose-dependent antagonism of the histamine-induced pressor effect, whilst peripheral administration of phentolamine (5 mg/kg i.v.) totally abolished this hypertensive response. Neither treatment significantly modified the positive chronotropic effect produced by histamine ($P > 0.05$).

6-Hydroxydopamine (3 x 250 μg i.c.v.) or mecamylamine (5 mg/kg i.v.) abolished both the pressor response and tachycardia to i.c.v. administered histamine, while atropine (100 μg i.c.v.) or propranolol (1 mg/kg i.v.) abolished the tachycardia without modifying the blood pressure rise. Acute bilateral vagotomy or adrenal demedullation failed to modify either cardiovascular change.

The results demonstrate that i.c.v. administration of histamine elicits blood pressure and heart rate increases in anaesthetized rats and are consistent with the view that centrally increased sympathetic nerve activity mediates these responses. Furthermore the central action of histamine appears to be mediated via histamine-H_1-receptors, although central adrenergic and cholinergic mechanisms also appear to be involved.

P.E.H. is the holder of an S.R.C. studentship in conjunction with Smith, Kline & French Laboratories.

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An anticholinergic effect of general anaesthetics on cerebrocortical neurones

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The effect of general anaesthetics on the increase in neuronal firing rate produced by the iontophoretic application of acetylcholine and L-glutamate is controversial. Krnjević & Phillis (1963) observed a specific anticholinergic effect (see also Catchglove, Krnjević & Maretić, 1972). But a nonspecific decrease in the post-synaptic sensitivity to all chemical excitants has also been reported (Crawford, 1970; Crawford & Curtis, 1966). It seemed worthwhile therefore to try to eliminate these discrepancies and possibly throw light onto the mechanism of action of general anaesthetics. Rats were anaesthetized with urethane (2.2 g/kg). Electrodes were placed on the exposed cortical surface for ECoG. recording and multibarrelled glass micropipettes inserted into the underlying cortical tissue for recording of single neuronal action potentials and the application of drugs by iontophoresis using the standard techniques.

Under urethane anaesthesia the ECoG. appears as surface positive waves separated by quiescent periods, with the spontaneous activity of cortical neurones occurring during these waves in the ECoG. (Bindman, Lippold & Redfearn, 1964). Monitoring the ECoG. therefore gives a measure of the endogenous drive to the cortex. The iontophoretic application of either L-glutamate or acetylcholine to responsive cortical neurones caused firstly an increase in the number of action potentials falling within the ECoG. waves (presumably representing facilitation of endogenous drive—Forrester, 1975) and then, if the rate of iontophoresis was sufficiently high, evenly spaced action potentials between the ECoG. waves.

When a variety of central depressants (including barbiturates, halothane and benzodiazepines) were systemically administered a decrease in the frequency of the ECoG. waves resulted-without changes in blood pressure (Forrester & Gartside, 1975). Consequently iontophoretically induced excitations which were largely dependent upon the drive to the cortex were reduced. In 5 experiments with halothane (1-2%, inspired) and 8 experiments with thiopentone (5-10 mg/kg, i.v.) both L-glutamate and acetylcholine excitations of this type were reduced.

When thiopentone (5-10 mg/kg, 9 experiments), halothane (1-2%, 12 experiments) and diazepam (1.5-2.0 mg/kg, 10 experiments) were administered to animals where, in the control responses both acetylcholine and L-glutamate induced action potentials between the bursts, then the production of 'interburst action potentials' by acetylcholine was suppressed but those produced by L-glutamate were not.

Hence it is concluded that the general anaesthetics tested antagonize the depolarization of cortical neurones produced by the iontophoretic application of acetylcholine but not that produced by L-glutamate; although depending upon the conditions the increase in neuronal firing rate produced by L-glutamate can be reduced. These results, therefore, support the view that the anticholinergic effect of general anaesthetics may contribute to their anaesthetic effect (Krnjević, 1974).

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The anticonvulsant activity of ketamine in mice following the inhibition of GABA synthesis by mercaptopropionic acid

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The dissociative anaesthetic, ketamine, has been reported to precipitate epileptiform discharges in the neocortex of the cat (Winters, Ferrar-Allado, Guzman-Flores & Alcaraz, 1972, Wong & Jenkins, 1974) although Celesia & Chen, (1974) have demonstrated that ketamine suppresses focal seizures in the same species. Similar conflicting evidence has been obtained in man (see Ferrar-Allado, Brechner, Dymond, Cozen & Crandall, 1973; Corssen, Little & Tavakoli, 1974). Ketamine is also known to inhibit glutamate decarboxylase (GAD) *in vitro* (Dye & Taberner, 1975) and therefore the effects of ketamine on the convulsions produced by another inhibitor of GAD, mercaptopropionic acid, have been examined.

All drugs were made up in physiological saline and injected i.p. into groups of 8 adult LACG mice of either sex. The time from injection to the first full tonic-clonic seizure was determined. The dose required to produce full seizures in all the mice (CD_{100}) was also determined.

Ketamine alone produced a dose-dependent loss of the righting reflex during which time the mice exhibited random twitching of the limbs. At a dose of 90 mg/kg the mice lost their righting reflex for 19.2 ± 1.8 minutes. Mercaptopropionate alone produced convulsions and running fits within 4 min at doses in excess of 20 mg/kg; the CD_{100} was 35 mg/kg. At this dose all the mice recovered within 30 minutes. The LD_{100} for mercaptopropionate was 140 mg/kg. When ketamine (90 mg/kg) was given simultaneously with the mercaptopropionate the minimum convulsive dose was increased to 168 mg/kg, the CD_{100} to 195 mg/kg and the LD_{100} to over 250 mg/kg. Ketamine did not affect the degree of inhibition of GAD observed *in vivo* following convulsive doses of mercaptopropionate. At the onset of convulsions after 150 mg/kg mercaptopropionate the inhibition was 35-39% compared to control mice. In mice given ketamine plus mercaptopropionate, which were not convulsing, the degree of inhibition was within the same range.

From these results it would appear that, despite the overt excitatory behavioural phenomena observed following hypnotic doses of ketamine, the latter can prevent seizures induced by mercaptopropionate. Also, at this dose, ketamine does not measureably inhibit GAD *in vivo* nor does it prevent the convulsive effects of mercaptopropionate by protecting GAD from inhibition. The results therefore support the view of Chen and his co-workers (Chen, Ensor & Bohner, 1966; Celesia & Chen, 1974) namely, that ketamine possesses anticonvulsant properties.

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Response of identified ventromedial hypothalamic nucleus neurones to putative neurotransmitters applied by microiontophoresis

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Recent physiological studies have indicated that the amygdala exerts a prominent excitatory influence on the majority of HVM neurones, and that HVM neurones have efferent connections to both hypothalamic (median eminence, anterior hypothalamic area) and extrahypothalamic (preoptic area, periaqueductal gray, amygdala and thalamus) areas (Renaud & Martin, 1975b). This study details preliminary results on the responsiveness of HVM neurones, identified through their afferent and efferent connections, to microiontophoretic application of substances which may act as neurotransmitter agents in this region.

Spike discharges from HVM neurones in pentobarbitone anaesthetized male Sprague Dawley rats were recorded with single 3.0 M NaCl filled micropipettes rigidly mounted to an adjacent multibarrelled micropipette filled with the following solutions:– monosodium L-glutamate (0.5 M, pH 7.0); gamma aminobutyric acid (0.5 M, pH 4.5); glycine (0.5 M, pH 3.5); histamine dihydrochloride (0.2 M, pH 4.0); dopamine HCl (0.2 M, pH 4.5); picrotoxin (5 mM, pH 7.5); strychnine sulfate (5 mM, pH 5.5); growth hormone release-inhibiting hormone or somatostatin (5 mM, pH 6.5); thyrotrophin releasing hormone (5 mM, pH 6.5); and luteinizing hormone releasing hormone (5 mM, pH 6.5).

The actions of these substances on the neural activity or excitability of HVM cells are summarized in Table 1.

Of the excitatory substances, the action of glutamate was brisk in onset and termination compared with the slow onset excitant action of histamine, an effect which outlasted the application by several seconds (Haas, 1974). The potent depressant action of GABA and glycine was rapid in onset and termination. Although these effects could be antagonized by picrotoxin and strychnine respectively, only picrotoxin applied either by microiontophoresis or by intravenous injection partially antagonized synaptic inhibition evoked by amygdala stimulation. Only depressant responses were observed with the three hypothalamic peptides tested. Selected HVM neurones displayed marked sensitivity to some peptides but not others (Renaud & Martin, 1975a, Renaud, Martin & Brazeau, 1975). The action of dopamine was never as potent as that of the depressant amino acids, but similar to the weaker depressant responses observed with some peptides.

More detailed study of HVM neurones with specific efferent connections, may indicate cellular populations with receptors for specific substances, and may help to define their biological significance in neural integration and regulation within this important hypothalamic centre.

Supported by the Canadian Medical Research Council.

Table 1 Pattern of response (no. of cells)

	Increase	*Decrease*	*No response*	*Current range (nA)*
Glutamate	46	0	0	24-80
Histamine	12	20	5	3-25
GABA	0	18	1	5-20
Glycine	0	16	1	8-22
Dopamine	0	18	6	20-50
GH-RIH	0	28	8	5-80
LH-RH	0	13	5	10-30
TRH	0	46	20	10-30

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Failure of denervation to influence the high affinity uptake of choline by sympathetic ganglia

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The transport process responsible for the uptake of choline in the central nervous system possesses high and low affinity components (Yamamura & Snyder, 1973; Haga & Noda, 1973, Neal & Gilroy, 1975) and there is some evidence that the high affinity choline transport process may be associated with cholinergic nerve terminals (Kuhar, Sethy, Roth & Aghajanian, 1973)

In the present study we have attempted to obtain further information on the possible association of the high affinity choline uptake process with cholinergic nerve endings by comparing the kinetics of choline uptake in normal and denervated ganglia.

Superior cervical ganglia were dissected from anaesthetized rats, de-sheathed, and then incubated for various times at 25°C in Krebs bicarbonate Ringer containing [^{3}H]-choline (1 μCi/ml). The ganglia were then dissolved in Soluene (Packard) and the radioactivity determined by liquid scintillation counting Unilateral decentralization of the ganglion was performed under halothane anaesthesia 12 to 17 days prior to excision of both ganglia.

Ganglia accumulated radioactivity when incubated with [^{3}H]-choline (1.8 x 10^{-7} M). This uptake was almost linear for 60 min at which time a tissue : medium ratio of approximately 9 was achieved. The effect of choline concentration on the uptake was studied over a concentration range of 0.2 to 600 μM. Since preliminary experiments indicated that uptake was linear over this concentration range for at least 20 min, incubations of this length were used to obtain estimates of the initial velocity of choline uptake. The kinetic data indicated that the uptake process for choline in ganglia was saturable and, as in brain, could be resolved into a high and low affinity process, with apparent Km values of 2.1 μM and 43 μM respectively The uptake of choline at both high and low concentrations was unaffected by denervation of the ganglia and a paired comparison of ganglia (7 pairs) at the lowest choline concentration (0.2 μM) revealed no significant difference between the choline uptake in denervated ganglia and the contralateral controls (Tissue; medium ratios = 3.20 ± 0.23 and 3.68 ± 0.31 respectively). It is unlikely that this result was due to failure of the denervation since ganglia were taken to be denervated only if there was obvious ptosis and a failure of the palpebral fissure to respond to bipolar stimulation (2 Hz, 0.5 ms, 30 V) of the preganglionic nerve stump. Denervated ganglia in the same series, showed greatly reduced ChAc activity. Furthermore, stimulation of denervated ganglia regionally superfused as described by Bowery & Tulett (1975) failed to increase the efflux of radioactivity from the soma although similar stimulation of control ganglia caused large increases in the release of radioactivity. These results imply that in ganglia the high affinity choline uptake process is not restricted to cholinergic nerve terminals.

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The spontaneous and electrically-evoked release of endogenous amino acids from the rat visual cortex

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The application of salt solutions containing 50 mM potassium ions onto the surface of the visual cortex of anaesthetized rats significantly increases the rates of release of endogenous taurine and γ-aminobutyric acid (GABA), (Clark & Collins, 1975). In the present study, the effect of electrical stimulation on the release of endogenous amino acids is reported.

Male rats of the Wistar strain were anaesthetized with urethane and samples collected from the exposed visual cortex using perspex cylinders (Mitchell, 1963) containing 50 µl of Ringer solution (NaCl, 139 mM; KCl, 4 mM; $NaHCO_3$, 2.1 mM; $CaCl_2$, 1.26 mM; $MgCl_2$, 1.15 mM, glucose, 0.339 mM; NaH_2PO_4, 0.189 mM; bubbled with 5% CO_2 in oxygen) which was changed every 10 minutes. The taurine, GABA, glycine, aspartate, glutamate, glutamine and alanine content of each sample was measured using a modification of the dansylation procedure originally described by Briel & Neuhoff (1972). Bipolar platinum electrodes (tip separation 1.0 mm) were inserted 0.5 mm into the brain surface either in the perspex cylinder, or 1 mm outside its rim or on the contralateral cortex. After 5 control samples, the surface of the brain was stimulated (100 Hz, 2.5 mA, 1.0 ms for 5 min) and samples collected during the following 80 minutes.

Table 1 shows the resting release of the amino acids. Transcallosal stimulation or electrical stimulation adjacent to the cup significantly increased the release of the inhibitory amino acids taurine and GABA but significantly reduced the release of the excitants glutamate and aspartate. However, when the electrode was sited in the cup, electrical stimulation significantly increased the release of taurine, GABA and glutamate, whereas that of aspartate was unaffected. The results will be discussed in the light of the suggested neurotransmitter roles for these amino acids.

Table 1 Release of endogenous amino acids from the visual cortex of the rat

		Release following stimulation (x resting)		
Amino acid	*Resting release (pmol min^{-1} cm^{-2}) (n = 13)*	*Electrode adjacent to cup (n = 7)*	*Transcallosal stimulation (n = 6)*	*Electrode in cup (n = 10)*
Taurine	179 ± 20	2.59 ± 0.49	2.89 ± 0.35*	3.59 ± 0.59
GABA	22 ± 3	2.47 ± 0.18*	3.19 ± 0.43*	9.0 ± 2.3*
Glycine	91 ± 7	1.43 ± 0.32	2.08 ± 0.46	1.21 ± 0.28
Aspartate	182 ± 18	0.47 ± 0.08*	0.48 ± 0.14*	1.44 ± 0.22
Glutamate	413 ± 41	0.63 ± 0.12*	0.37 ± 0.06*	4.9 ± 0.69*
Glutamine	735 ± 70	1.17 ± 0.26	1.59 ± 0.29	1.28 ± 0.21
Alanine	98 ± 8	1.16 ± 0.24	1.19 ± 0.20	1.62 ± 0.18

In those experiments in which the preparation was stimulated transcallosally, or in which the electrode was adjacent to the cup, the mean of each group of 5 prestimulation samples was used to calculate the resting release of each amino acid as shown in the Table. Following stimulation, the maximum change in release in each experiment was identified and expressed as a multiple of the mean resting release of that experiment. In the other experiments, because the surface of the brain within the cup was punctured by the electrode, a different resting release was obtained (not shown) and used in calculating the results.

Each value is a mean ± s.e. mean. *n* = number of observations. An asterisk denotes a significant difference (Students *t* test) between the appropriate resting and evoked release ($P < 0.05$).

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The actions of GABA on d.c. and field potential recordings from the rat cuneate nucleus

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GABA is thought to be a presynaptic inhibitory transmitter in mammals largely because the GABA antagonists picrotoxin and bicuculline block presynaptic inhibition (Eccles, Schmidt & Willis, 1963, Levy, Repkin & Anderson, 1971). The present experiments are an attempt to demonstrate that exogenous GABA will enhance or mimic presynaptic inhibition, as previous attempts to do this have only been partially successful (Curtis & Ryall, 1966; Schmidt, 1971).

Experiments were performed on 20 rats prepared as described previously (Collins, Hill & Roberts, 1975). In addition, d.c. potentials were recorded from a micropipette inserted into the cuneate nucleus. The exposed dorsal surface of the medulla was continuously superfused with a Krebs bicarbonate solution to which solutions of amino acids were added. Changes in the N- and P-waves of the cuneate field potential were measured from computer averaged records.

GABA (0.01-0.5 M) in the superfusion solution reduced the size of the N-wave and increased the size of the P-wave (Figure 1) This was dose-related and approximately linear over the range 0.02-0.1 M. Microiontophoretic application of GABA (25-150 nA) produced similar changes in the field potentials. A negative d.c. shift coincided with the increase in the P-wave (Figure 1) and this was also dose-related. The GABA-evoked d.c. shift increased with depth in the medulla reaching a maximum at about the inversion point of the P-wave, and declining thereafter without inversion This maximum coincides with the greatest density of primary afferent terminals within the cuneate and the d.c. shift may thus be a reflection of the depolarization of these terminals associated with the generation of the P-wave (Andersen, Eccles, Schmidt & Yokota, 1964). Replacement of Ca^{++} in the Krebs solution with 20 mM Mg^{++} and 0.5 mM EGTA attenuated the synaptically evoked field potential (<40% control) but did not reduce the d.c. shift suggesting that GABA may be acting directly rather than transynaptically.

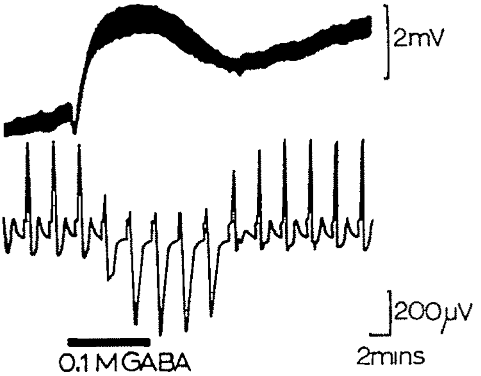

Figure 1 D.c. (upper) and computer averaged (16 sweeps) field potential records (lower) from rat cuneate nucleus in response to electrical stimulation of the ipsilateral forepaw. Application of GABA produced a negative d.c. shift accompanied by a decrease in the size of the N-wave and an increase in the P-wave. Negativity upwards in both records.

Although these results support the idea that GABA could be a presynaptic inhibitory transmitter this is not conclusive as glycine, β-alanine and taurine have been found to produce similar field potential and d.c. changes in the cuneate nucleus.

This work is supported by a grant from the M.R.C.

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Bimodal effect of glycine on frog spinal motoneurones

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Glycine has been shown to have variable effects on the polarity of amphibian motoneurones as recorded from ventral roots of the isolated hemisected spinal cord (Tebecis & Phillis, 1969, Barker & Nicoll, 1973). In the present experiments regenerative activity in isolated frog hemicords was blocked with procaine which allowed the direct effect of glycine on the polarity of motoneurones to be measured. In such preparations it was found that glycine evoked either hyperpolarization or depolarization depending on the composition of the Ringer solution bathing the cord.

The standard Ringer solution contained (mM):– NaCl, 111; KCl, 2; $CaCl_2$, 2; Tris base, 10; glucose, 12; procaine HCl, 1; adjusted to pH 7.9 with HCl. With this solution, glycine (0.25-2 mM) regularly produced depolarization. Chloride-deficient Ringer ($NaMeSO_4$, 111 mM; $KMeSO_4$, 2 mM; $CaSO_4$, 2 mM) enhanced the depolarization caused by glycine. However, in potassium deficient (KCl, 0.5 mM) and sodium deficient (NaCl, 22 mM; choline chloride, 88 mM) Ringer solutions the depolarization caused by glycine was diminished and hyperpolarization often occurred. Glycine also produced hyperpolarization when tested in the presence of a depolarizing concentration of L-glutamate (0.2-0.5 mM) The reversal of the response to glycine produced by L-glutamate could not have been due to depolarization alone because similar depolarizations produced by potassium chloride (4-10 mM, total KCl) did not cause glycine to hyperpolarize.

Strychnine (50 μM) increased glycine-induced depolarizations and converted glycine-induced hyperpolarizations into depolarizations.

Thus it appears that glycine has a strychnine-sensitive hyperpolarizing action and also a depolarizing action which may be mediated by different receptors on the motoneurone. It is probable that the hyperpolarizing receptor is activated by β-alanine and taurine in addition to glycine because these amino acids also have hyperpolarizing actions which are blocked by strychnine.

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Extracellular and intracellular effects of procion brilliant red H3BN on mammalian muscle fibres

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The interpretation of the physiological and pharmacological properties of muscles from animals suffering from the degenerative diseases of muscle is hindered by the occurrence of severely damaged and normal fibres within the same muscle. One approach to overcoming this obstacle would be to mark individual fibres with a stable dye during an experiment and subsequently to isolate these fibres for morphological and histochemical examination.

The Procion dyes (Stead, 1973), and particularly Procion Yellow, have been widely used for marking nerve cell processes, but not extrafusal muscle fibres. Procion dyes are known to bind to cellular protein (Flanagan, Hesketh & Chung, 1974) but the possibility that they may alter the physiological characteristics of the exposed or injected cells has not been examined. It has, however, been suggested that Procion Yellow has local anaesthetic properties (Payton, 1970).

We have examined the effects of extracellular and intracellular Procion dyes on some properties of mammalian muscle. Procion Yellow MX4R, Procion Navy Blue H3R and Procion Brilliant Red H3BN have all been tested, but only Procion Brilliant Red was found to be routinely suitable for intracellular injection.

Experiments were carried out on isolated extensor digitorum longus, soleus and diaphragm muscles of normal rats and mice. The muscles were maintained *in vitro* at room temperature. The presence of Procion Brilliant Red (1% w/v; -5×10^{-3} M) in the bath had no effect on membrane potential, miniature end-plate potentials, 'input resistance', or action potentials elicited either by indirect stimulation or direct excitation of individual muscle fibres, 1 h after its application.

In another series of experiments individual muscle fibres were injected with Procion Brilliant Red by passing hyperpolarizing direct currents of 40 nA through an intracellular micropipette containing a 4% (w/v) solution of the dye. In these experiments the dye-filled micropipette was used as the current passing electrode when measuring 'input resistance' or generating muscle fibre action potentials. Action potentials were measured immediately following penetration of the dye-filled micropipette and again 1-2 min later.

In many fibres dye injection was associated with a localized swelling of the muscle fibre and sometimes this was accompanied by a rapid fall in membrane potential. In most fibres, however, there was no significant change in any measured parameter. Satisfactory marking of the muscle fibres was attained within 15-20 s of penetration.

The results suggest that Procion Brilliant Red H3BN is a suitable agent for marking individual muscle fibres, and within the context of these experiments, is devoid of appreciable pharmacological activity.

Details of the techniques used to prepare the micropipettes, and of the techniques of injection and post-injection fixation are described elsewhere (Harris & Ribchester, 1975).

This work was supported by grants from the Muscular Dystrophy Associations of America Inc. and the Muscular Dystrophy Group of Great Britain. R.R.R. is in receipt of an M.R.C. Studentship for training in research methods.

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The mechanism of 'adrenaline reversal' in the anaesthetized cat and rabbit

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α-Adrenoceptor blockers share the ability to produce adrenaline reversal in anaesthetized laboratory animals (Dale, 1906; Nickerson, 1949). Adrenaline activates vasoconstrictor (α-) and positive inotropic and chronotropic (β_1-) adrenoceptors which mediate pressor responses, and vasodilator (β_2-)adrenoceptors which are depressor. Therefore, α-blockade will only produce adrenaline reversal if the β_2-effect transcends the β_1-action. This could result from three possibilities:— (1) the β_2-action intrinsically exceeds the β_1-effect; (2) the β_2-response is potentiated by α-blockade; (3) the α-blocker antagonizes the β_1-action.

We examined these hypotheses in 41 adult cats anaesthetized with i.p. chloralose (80 mg/kg) or sodium pentobarbitone (35 mg/kg). In 17 cats, by selective blockade with phentolamine, practolol or propranolol, we determined the contributions of α-, β_1-, and β_2-adrenoceptor responses to the BP changes elicited by i.v. noradrenaline, adrenaline or isoprenaline doses up to 10 μg/kg. The log dose-response curves showed for adrenaline:— firstly, the β_1-threshold is highest; secondly, the β_2-effect often predominates at low doses giving depressor actions but at higher doses the α- and β_1-effects cause a pressor response; thirdly, at all doses and in all cats the β_2-action exceeds the β_1-effect. The α- and β_1-components of noradrenaline are similar to those of adrenaline but β_2-activation is weak (see later). Isoprenaline shows β-effects greater than those of adrenaline: but the α-blocker left the isoprenaline response unchanged which indicates that phentolamine has little significant action upon β-components. In further experiments, using ten different α-blockers, we found that neither β_1-depression nor β_2-potentiation provides a satisfactory explanation for adrenaline reversal.

Adrenaline reversal is invariably associated with a marked degree of vasodilator (β_2-) stimulation.

This activation also explains why noradrenaline is more pressor than adrenaline in the cat—their α- and β_1-actions are similar but usually (see later) only adrenaline has an appreciable depressor (β_2-) influence. Corroborative signs of substantial β_2-adrenoceptor stimulation are the depressor effects of low doses of adrenaline and secondary hypotensive phases following the initial pressor responses to higher doses. With noradrenaline, the above signs only occurred, and even then infrequently, in three cats and one rabbit (see later). In these animals alone noradrenaline reversal sometimes happened (Karim, 1964).

In the rabbit, adrenaline reversal is uncommon (Harvey & Nickerson, 1953) yet β_2-effects (diastolic BP falls) undoubtedly occur with isoprenaline. In 5 out of 8 New Zealand White rabbits (i.p. urethane, 2 g/kg), the β_2-activation produced by adrenaline was negligible and adrenaline and noradrenaline were equipressor. But, in the other 3 rabbits, adrenaline had a stronger β_2-action so that low doses were depressor and pressor effects less than those of noradrenaline: these rabbits were the only ones to show adrenaline reversal

Therefore, in both cats and rabbits, 'adrenaline reversal' is invariably linked with the extent of β_2-adrenoceptor stimulation, and is explicable solely as a phenomenon of α-adrenoceptor blockade unveiling a β_2-action which intrinsically exceeds the corresponding β_1-effect.

Financial support from the Wellcome Trust is gratefully acknowledged.

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45-Calcium uptake in rat peritoneal mast cells

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When sensitized rat peritoneal mast cells are challenged with antigen, the resulting secretion of histamine is dependent upon calcium in the external medium (Foreman & Mongar, 1972). Calcium entry into the cell appears to provide the trigger for histamine release (Foreman, Mongar & Gomperts, 1973). Indirect evidence suggests that calcium entry as well as histamine release is blocked by dibutyryl cyclic AMP (Foreman, Mongar, Gomperts & Garland, 1975).

Changes in the amount of calcium associated with the cell have been measured in a purified suspension of rat peritoneal mast cells from Lister Hooded rats, sensitized with egg albumin and *Bordetella pertussis* adjuvant 2-3 weeks previously. The cells were purified by centrifugation through a human serum albumin gradient (20% and 26% albumin). The pellet contained 70-90% mast cells. After washing, the cells were incubated for 5 min in 100 µl Tyrode solution, containing 45-calcium chloride, above 100 µl silicon oil in a micro-centrifuge tube. The tubes were spun for 30 s at 15,000 *g* to separate the cells from the radioactive medium. The pellet, after removal by freezing and cutting off the bottom of the micro-centrifuge tube, was dissolved in Triton-X—100 and the radioactivity measured by liquid scintillation counting.

On average antigen caused 94 nmol histamine release and 0.3 nmol calcium uptake per million cells. With unstimulated cells the corresponding values were 28 nmol histamine release and 0.55 nmol calcium uptake. When pre-incubated with metabolic inhibitors, antimycin A and cyanide, histamine release was abolished but calcium uptake was only slightly reduced (Figure 1). In the absence of inhibitors part of the calcium uptake is possibly due to the increase in surface area of the cells that accompanies histamine release. When this is inhibited a small inhibition of calcium uptake may occur. With dibutyryl cyclic AMP both histamine release and 45-calcium uptake were inhibited.

These results suggest that the increase in 45-calcium associated with cells after antigen challenge is due to an increase in membrane permeability to calcium. Opening of the calcium

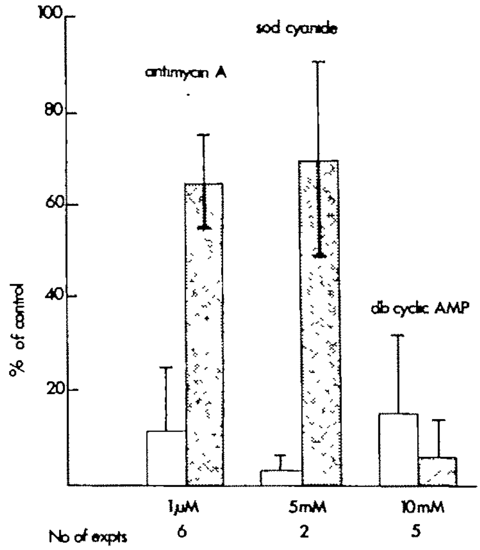

Figure 1 The effect of metabolic inhibitors and dibutyryl cyclic AMP on 45-calcium uptake (hatched columns) and histamine release (open columns). Vertical bars show standard error of mean.

channels is not dependent upon ATP production, but appears to be opposed by cyclic AMP. Further evidence for this mechanism is provided by the correlation (r = 0.85, *n* = 14) between cell sensitization (measured by uninhibited histamine release, 5-35% of cell content) and 45-calcium uptake.

MBH thanks the MRC for a training award.

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Reserpine-induced supersensitivity to the rate and tension responses to isoprenaline and salbutamol of guinea-pig atria

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Reserpine, as well as depleting neuronal catecholamines produces a non-specific supersensitivity (Trendelenburg, 1963). This has been demonstrated in isolated cardiac preparations where both the positive inotropic (McNeill & Schulze, 1972) and chronotropic responses (Westfall & Fleming, 1968) to noradrenaline were potentiated by reserpinization. However, Crout, Muskus & Trendelenburg (1962) failed to produce supersensitivity to the rate responses and Taylor, Westfall & Fleming (1974) have recently claimed that it is only the rate responses that are potentiated. To clarify these divergences of opinion, we have examined the effect of a range of reserpine pretreatment regimes upon both the inotropic and chronotropic responses to isoprenaline as the agonist.

Separated left and right guinea-pig atria were suspended in Krebs-bicarbonate solution at 38°C gassed with CO_2 : O_2, 5%:95%. The left atrium was paced electrically at 2.5 Hz and isometric tension recorded on a Devices M19 polygraph for the inotropic responses. Chronotropic responses were obtained by means of a ratemeter triggered by the isometric tension signal of the spontaneous right atrium. Two cumulative dose response curves to isoprenaline were obtained and the second used for plotting purposes. In some experiments comparisons were made between this curve and a third curve to another agonist.

Isoprenaline in untreated atria was more selective for rate in that the rate dose response curve was to the left of that for tension. Isoprenaline was then examined in atria taken from guinea-pigs receiving the following reserpinization schedules; (a) 5 mg/kg at 72 h, 3 mg/kg at 48 h and 3 mg/kg at 24 h, (b) 5 mg/kg at 24 h and (c) 0.5 mg/kg at 24 h before sacrifice. Both the rate and tension dose response curves were displaced to the left by the three day pretreatment (a) suggesting the possibility of supersensitivity. However, the tension curve was shifted more, so that the selectivity for rate previously seen in untreated atria was reduced. As the severity of the reserpinization became less, so the supersensitivity declined and the separation of rate and tension curves became more apparent and comparable with the untreated situation. To avoid supersensitivity to reserpine we therefore used dosage regime (c). This dosage, however, satisfactorily depleted catecholamines since the indirectly acting sympathomimetic β-phenylethylamine was virtually ineffective in a dose that produced a significant response in untreated atria. The supersensitivity to schedule (b) was found to be non-selective since it was also demonstrated to histamine.

Salbutamol, a sympathomimetic amine having partial agonist properties in the heart (Brittain, Jack & Ritchie, 1970) was then compared with isoprenaline. In untreated atria salbutamol was almost a full agonist on rate but only a partial agonist on the tension with a mean maximum response only 10.8% (± 2.0%, $n = 4$) that of isoprenaline. However, in atria from animals receiving treatment (b) and (a), the maximum tension response was raised to 24.0% (± 6.8%, $n = 4$) and 52.5% (± 9.61, $n = 4$) respectively.

This study therefore demonstrates non-selective supersensitivity to isoprenaline by reserpinization on both rate and tension, the latter being more pronounced. Furthermore, a partial agonist on tension can be progressively shifted towards full agonist activity.

This work was supported jointly by a CAPS grant from SRC and Allen & Hanburys Ltd.

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Is ATP an inhibitory neurotransmitter in the rat stomach?

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Burnstock (1972) has postulated a neurohumoral role for ATP or a related nucleotide in 'purinergic' inhibitory nerves of the gastrointestinal tract.

The rat stomach has been shown to contain non-adrenergic inhibitory neurones from which release of the transmitter substance could not be demonstrated. After a wide range of potential neurotransmitters had been examined, only ATP and adenosine merited further investigation (Heazell, 1974). The hypothesis that the rat stomach contained purinergic inhibitory fibres was studied pharmacologically by comparing the effect of certain drugs upon the responses to exogenous ATP and adenosine with those to nerve stimulation.

A rat fundal strip (Vane, 1957) was placed in oxygenated Krebs solution at 32°C containing hyocine (1×10^{-6} M), 5-HT (3×10^{-7} M) to maintain tone and sodium metabisulphate (5×10^{-3} M) as an antioxidant. Sequential dose-response curves were obtained to ATP and adenosine.

The nature of response to a low dose of ATP (1×10^{-6} M) was not consistent although with higher doses up to 2×10^{-3} M, small relaxations were obtained sometimes followed by a contraction. Adenosine (1×10^{-5}-1×10^{-4}M) also caused a variety of effects, higher doses resulting in relaxation.

Dipyridramole and hexobendine (Satchell, Lynch, Bourke & Burnstock, 1972) which have been shown to potentiate 'purinergic' transmission increased the inhibitory response to exogenous purine. By contrast, responses to field stimulation with pulses of 30 V cm^{-1} (measured in Krebs solution), 0.2 ms duration at frequencies to produce maximal and sub-maximal effects were slightly reduced by dipyridamole and not significantly different following hexobendine.

2-2′ Pyridylisatogen tosylate, a specific antagonist of ATP (Hooper, Spedding, Sweetman & Weetman, 1974), reduced the inhibitory effects of ATP and adenosine. The response to electrical stimulation was reduced but was not significantly lower than the control; some of this reduction was due to the decline in muscle tone following addition of drug.

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On the basis of these observations, the non-adrenergic inhibitory innervation of the rat stomach is unlikely to be purinergic.

I am grateful for the following gifts of drugs; dipyridamole (Boehringer-Ingelheim Co. Ltd.) hexobendine (Osterreichische Stickstoffwerke A.G) and 2-2'pyridylisatogen tosylate (Dr D.F. Weetman, School of Pharmacy, Sunderland Polytechnic).

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Comparison of the effects of selective α- and β-receptor agonists on intracellular cyclic AMP levels and glycogen phosphorylase activity in guinea-pig liver

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(–)-Isoprenaline (a selective β-receptor agonist) and (±-amidephrine (a selective α-receptor agonist) have been shown to increase glucose release from guinea-pig liver slices (Haylett & Jenkinson, 1972a,b). The effects of these agents on intracellular cyclic AMP levels and glycogen phosphorylase activity have now been measured under similar experimental conditions. The slices were exposed to the agonists for 2 min, at which time increased glucose release was substantial but not maximal. Cyclic AMP was determined by a protein binding technique (Tovey, Oldham & Whelan, 1974) in samples extracted from slices by TCA precipitation of acid insoluble material, after freezing the tissue in liquid nitrogen. The supernatant was passed through a 3.0 x 0.5 cm column of Dowex 50 (H^+ form) to remove the TCA and substances that might interfere with the cyclic AMP assay. Glycogen phosphorylase activity was measured by the method of Danforth, Helmreich & Cori (1962). Glucose release was measured by the procedure of Park & Johnson (1949).

Doses of agonist which cause near maximal glucose release—isoprenaline 20 nM, amidephrine 20 μM—both produce comparable increases in phosphorylase activity. However while the cyclic AMP level in slices treated with this dose of isoprenaline was significantly greater than the control values ($P < 0.05$), there was little change in the cyclic AMP level in slices treated with amidephrine (Figure 1). Glucose release in the 2 min exposure to amidephrine was 53.8 ± 11.8% ($n = 16$) above control release, as compared with 25.9 ± 8.1% ($n = 15$) after isoprenaline. The increase in phosphorylase activity was shown to be dose-related between 4 and 20 μM amidephrine. The response to 20 μM amidephrine, but not that to 20 nM isoprenaline, was abolished in the presence of 40 μM phentolamine.

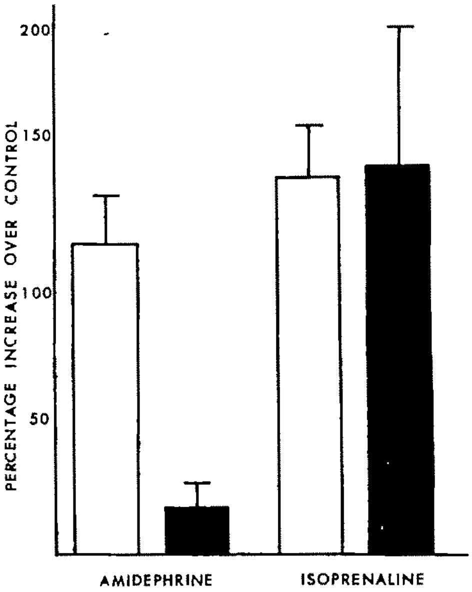

Figure 1 Effect of amidephrine (20 μM) and isoprenaline (20 nM) on glycogen phosphorylase activity (□) and tissue level of cyclic AMP (■). Each histogram represents the mean of at least 7 experiments; the vertical bar represents one s.e. mean.

These results suggest that amidephrine activates glycogen phosphorylase by an α-receptor mediated mechanism not associated with a rise in the intracellular level of cyclic AMP.

I note the receipt of an MRC Training award, and thank Mrs M. Butler for her excellent technical assistance.

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Cyclic AMP in HeLa cells stimulated with cholera enterotoxin and methylxanthines

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There are two general approaches being used to elucidate the regulatory role of cyclic AMP in cell growth. One is to add cyclic AMP or its analogues to the system under study and observe its effects upon various parameters of cell activity. The other approach is to alter the intracellular levels of cyclic AMP by modulation of adenyl cyclase or phosphodiesterase activity. We report here our attempts to manipulate endogenous cyclic AMP in cells in tissue culture.

HeLa cells were grown in suspension culture in Jokliks medium containing 7% calf serum to a density of 8×10^5 cells/ml. Cyclic AMP levels were measured as described previously (Albano, Barnes, Maudsley, Brown & Ekins, 1974). In cells incubated in the presence of 8 mM theophylline cyclic AMP levels were increased by cholera enterotoxin (1 μg per 10^6 cells) but not by prostaglandin E_1 (10 μg) or adrenaline (10 μg). In the absence of theophylline, toxin produced little or no increase in cyclic AMP. A characteristic of toxin stimulation of cyclic AMP is the existence of a lag period between the addition of the toxin and the increase in cyclic AMP (Pierce, Greenough & Carpenter, 1971). In HeLa cells the lag period is of 5-10 min duration which is much shorter than it is for several other systems such as adrenal cells (Haksar, Maudsley & Péron, 1975).

Theophylline alone in concentrations up to 32 mM or 3-isobutyl-1-methylxanthine in concentrations up to 2 mM did not increase the basal levels of cyclic AMP. These concentrations of theophylline and 3-isobutyl-1-methylxanthine, however, did reduce the uptake of $[^3H]$-leucine. Caffeine had similar effects and of the agents used 3-isobutyl-1-methylxanthine was the most potent and caffeine the least active. Thus, while the relative potencies of the methylxanthines approximate their known ability to inhibit cyclic nucleotide phosphodiesterases the effects upon transport may not be mediated by cyclic AMP. In this system, therefore, methylxanthines do not appear to be useful for the manipulation of basal cyclic AMP levels. Furthermore, these results also indicate that in HeLa cells cyclic AMP produced in response to toxin is turned over more rapidly than cyclic AMP in the resting cell.

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Comparison of circling behaviour following unilateral inhibition of GABA-transaminase or discrete electrolytic lesioning in the rat substantia nigra

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The concentration of GABA can be elevated unilaterally in the rat substantia nigra by local injection of ethanolamine-*O*-sulphate (EOS) (Dray, Oakley & Simmonds, 1975) an irreversible active-site-directed inhibitor of GABA-transaminase (GABA-T) (Fowler & John, 1972, Fowler, 1973). In such animals both apomorphine and amphetamine have been shown to produce *ipsilateral* turning behaviour (Dray *et al.*, 1975). We wish to report further correlates of motor activity changes following discrete unilateral electrolytic lesions or unilateral elevation of endogenous GABA concentration in one substantia nigra.

Male albino rats (200-250 g) were anaesthetized with halothane and unilateral injections of EOS (200 μg kg^{-1} in 1.5 μl) were made stereotaxically into the substantia nigra:zona reticulata (SNR). Discrete unilateral electrolytic lesions (1 mA for 20 s) were made either in the substantia nigra : zona compacta (SNC) or in the SNR. Both injection and lesion sites were confirmed histologically.

Animals with EOS injected into the SNR showed tight ipsilateral circling behaviour after an i.p. injection of apomorphine (4 mg kg^{-1}) or amphetamine (4 mg kg^{-1}). This was observed at 4-6 h after the EOS injection in some animals and by 24 h in all animals. The intensity of circling decreased after 3-5 days and was absent after 10 days.

The circling behaviour was associated with a significant reduction in GABA-T activity and an elevated GABA concentration in the injected SNR compared with the contralateral side. There was a significant decrease in ipsilateral striatal dopamine content but no consistent differences in striatal noradrenaline. By contrast, animals injected with EOS above the substantia nigra showed no circling behaviour after apomorphine or amphetamine and SNR GABA-T activity and GABA concentration was normal.

Unilateral electrolytic lesioning in the SNC produced the expected contralateral turning after apomorphine and ipsilateral turning after amphetamine (Ungerstedt, 1971a,b). However, after discrete lesions in the SNR, animals exhibited *ipsilateral* circling when challenged with either apomorphine or amphetamine. These responses could still be consistently produced 7 weeks after lesioning.

In both EOS injected and electrolytically lesioned animals, circling behaviour in the same direction as that produced by apomorphine could also be produced by i.p. injections (10-50 mg kg^{-1}) of the dopamine-receptor stimulant bromocriptine (Corrodi, Fuxe, Hökfelt, Lidbrink & Ungerstedt, 1973). However, this compound had a more prolonged onset and duration of action than apomorphine and circling behaviour was generally less intense

These results suggest that similar changes in induced locomotor activity may be produced by two different experimental procedures, i.e. the elevation of GABA concentration or discrete electrolytic lesions in one SNR. These changes may be tentatively accounted for by postulating an ipsilateral increase in ascending nigro-striatal dopaminergic neurotransmission. This could result from the reduced activity of non-GABA inhibitory interneurones within the SNR either by their GABA-mediated inhibition following EOS, or by their electrolytic destruction.

We thank Sandoz Products Limited for a gift of bromocriptine. The work was supported by an M.R.C. Grant to D.W.S. N.R.O. is an M.R.C. Scholar. T.T. is supported by a Pfizer studentship.

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Differences in circling responses following electrolytic and 6-hydroxydopamine lesions of the nigro-striatal pathway

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After injection of 6-hydroxydopamine into one substantia nigra to destroy the nigrostriatal dopaminergic neurones, rats circle towards the side of the lesion when amphetamine is administered systemically (due to release of dopamine from the opposite intact nigrostriatal pathway), and away from the side of the lesion when given apomorphine (due to preferential stimulation of presumed supra-sensitive dopamine receptors in the denervated striatum) (Ungerstedt, 1971). However, if an electrolytic lesion is placed in one nigrostriatal pathway, rats circle towards the side of the lesion when either apomorphine or amphetamine is administered (Costall & Naylor, 1975). We have therefore examined the biochemical and behavioural differences between a unilateral 6-hydroxydopamine lesion and an electrolesion of the nigrostriatal pathway. The results are summarized in the Table.

Unilateral injection of 6-hydroxydopamine (8 μg in 4 μl) just anterior to substantia nigra (A 30, V–2 7, L±2.0; De Groot, 1959), or into the medial forebrain bundle at the level of the lateral hypothalamus (A 4.6, V – 2.7, L ± 1.9), caused a profound reduction in ipsilateral striatal dopamine concentration. The nigral injection had no effect on cortical noradrenaline or striatal 5-hydroxytryptamine. The medial forebrain bundle lesion but not the nigral lesion caused a fall in cortical noradrenaline. Both lesions resulted in contraversive rotation to s.c. apomorphine and ipsiversive rotation to i.p. amphetamine. The medial forebrain bundle lesion caused enhanced contralateral postural deviation and circling to dopamine (50 to 100 μg in 1 μl) injected into the denervated striatum.

Unilateral electrolesions of substantia nigra (A 2.2, V – 1.7, L ± 2.0) or medial forebrain bundle at the level of the lateral hypothalamus (A 4.6, V – 2.7, L ± 1.9) caused changes in forebrain monoamine concentrations, including a considerable drop in ipsilateral striatal dopamine. But these electrolesions caused ipsiversive rotation to both s.c apomorphine and i.p. amphetamine (the threshold dose of apomorphine required being

Table 1 Comparison of effects of unilateral 6-hydroxydopamine and electrolytic lesions on forebrain monoamines and drug-induced circling behaviour

	6-hydroxydopamine		*Electrolesion*	
	Substantia nigra	*Medial forebrain bundle*	*Substantia nigra*	*Medial forebrain bundle*
		*Forebrain monoamine concentrations**		
Striatal dopamine	$29\%^{c}$	$18\%^{c}$	$25\%^{c}$	$57\%^{a}$
Cortical noradrenaline	83%	$63\%^{b}$	103%	$67\%^{a}$
Striatal 5-hydroxytryptamine	91%	91%	90%	83%
		Effect of systemic administration of drugs†		
Amphetamine	I 0.63	I 0.63	I 1.25	I 1.25
Apomorphine	C 0.03	C 0.015	I 0.125	I 0.125
		Effects of intrastriatal dopamine‡		
	—	Marked contraversive posture or circling	Nil	—

* Forebrain monoamine concentrations ipsilateral to the side of the lesion are expressed as a per cent of values in the opposite intact side.

† The direction of circling behaviour is indicated by I = towards the side of the lesion, and C = away from the side of the lesion. The threshold dose of drug (in mg/kg, i.p. or s.c.) required to produce such circling is also shown.

‡ Direct intrastriatal injection of 100 μg of dopamine into one intact striatum produced periodic contralateral postural asymmetry but no circling. The effect of injection of dopamine (50-100 μg) into the denervated striatum is shown

Superscripts indicate significance (Student's *t* test of paired samples) a = $P < 0.01$, b = $P < 0.005$, c = $P < 0.001$.

more than four-fold greater than that required to cause contraversive rotation after 6-hydroxydopamine lesions). The substantia nigra electrolesion abolished the effects of intrastriatal dopamine.

If an electrolesion in substantia nigra was followed by a 6-hydroxydopamine injection into the medial forebrain bundle, unilateral striatal dopamine fell to 33% of normal. Such animals rotated only ipsiversively to both s.c apomorphine and i.p. amphetamine.

An electrolesion in substantia nigra (or medial forebrain bundle) appears to abolish the effect of s.c. apomorphine or intrastriatal dopamine on the denervated striatum. We conclude that such electrolesions destroy not only the ascending nigrostriatal dopaminergic pathway, but also a second neuronal system, perhaps a non-dopaminergic nigrostriatal tract or a strio-pallidal efferent pathway, required for expression of rotational behaviour resulting from stimulation of dopaminergic receptors in the denervated striatum.

Drs B.C. and R.J.N. acknowledge support from the Medical Research Council. C.P. is a Fellow of the Parkinson's Disease Society.

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The role of dopamine in rotational behaviour produced by unilateral lesions of the locus coeruleus

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Rats with unilateral electrolytic lesions of the locus coeruleus rotate contraversive to the side of the lesion when given apomorphine hydrochloride (1 mg/kg i.p.) or dexamphetamine sulphate (5 mg/kg i.p.). This turning is transient and persists for less than 2 months. It is accompanied by a rise in the dopamine content of the ipsilateral striatum which is also transient and has a similar time course to the turning (Marsden & Pycock, 1974; Pycock, Donaldson & Marsden, 1975). Further experiments suggest that it is due to asymmetrical stimulation of the striatal dopamine receptors.

The turning induced in such animals by dexamphetamine is not modified by pretreatment with alpha (phenoxybenzamine 20 mg/kg i.p. 90 min before) or beta (propanolol 5 mg/kg i.p. immediately before) adrenoceptor blockers. However, turning to both dexamphetamine and apomorphine is completely abolished by pretreatment with the dopamine receptor blocker pimozide (0.25 mg/kg i.p. 4 h before). Similarly the adrenoceptor stimulant clonidine (0.05-0.5 mg/kg i.p.) does not produce rotation whereas the dopamine receptor stimulant piribedil (100 mg/kg i.p.) does.

Rotation produced by a locus coeruleus lesion is abolished by a unilateral electrolytic lesion of the substantia nigra on the same side. The resultant turning is the same as that normally produced by the substantia nigra lesion alone.

Intrastriatal injections in rats with unilateral locus coeruleus lesions show that the striatum on the side of the locus lesion is more sensitive to apomorphine than the opposite striatum. The dose of apomorphine required to produce rotation when injected intrastriatally on the side of the lesion was only 22 ± 6 micrograms while 61 ± 9 micrograms was required to produce the same effect when injected into the opposite striatum.

These results are consistent with the suggestion that the drug induced turning behaviour due to a unilateral locus coeruleus lesion in the rat is mediated via the ipsilateral striatal dopamine receptors. Their behaviour may be altered by the locus lesion which may have a facilitatory effect on transmission in the ipsilateral nigrostriatal pathway.

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Action of LSD on supersensitive mesolimbic dopamine receptors

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Since Ungerstedt & Arbuthnott (1970) described the amphetamine-induced rotation of rats with unilateral 6-hydroxydopamine (6-OHDA) lesions of the substantia nigra this *in vivo* preparation has been widely used to study the effects of drugs on dopaminergic mechanism in the brain. Recently it has been shown that LSD, like the dopamine agonist apomorphine, produces rotation towards the unlesioned side (Pieri, Pieri & Haefely, 1974) and it was suggested that LSD can act as a dopamine agonist. Since 6-OHDA lesions of the substantia nigra which destroy the nigrostriatal bundle also damage the mesolimbic dopaminergic innervation of the nucleus accumbens and olfactory tubercle we have examined whether this damage to the mesolimbic dopamine system is involved in the production of rotational behaviour.

Adult male Sprague Dawley albino rats were injected unilaterally with 8 μg of 6-OHDA into either the caudate nucleus or the nucleus accumbens septi. The 6-OHDA (hydrobromide) was dissolved in 2 μl of cooled saline containing 1 mg/ml ascorbic acid and injected at the rate of 1 μl/min. The caudate injection depleted striatal dopamine (DA) content by 76% without significantly affecting the concentration of DA in the nucleus accumbens or olfactory tubercle. The nucleus accumbens injection reduced the DA content of the nucleus accumbens by 91% and that of the olfactory tubercle by 78%, and caused a smaller (24%) reduction in striatal DA content. When injected with (+)-methamphetamine (5 mg/kg i.p.) or apomorphine (10 mg/kg i.p.) 10-14 days after the lesion, only the caudate-lesioned rats showed a large rotational response to these drugs. The rotation produced by LSD is therefore probably due to an action on striatal dopamine receptors.

To investigate whether LSD can act as an agonist at mesolimbic dopamine receptors we recorded its effect in rats with bilateral 6-OHDA lesions of the nucleus accumbens. These animals show a greatly enhanced stimulation of locomotor activity when injected with dopamine agonists such as apomorphine (Kelly, Seviour & Iversen, 1975) or N-*n*-propylnorapomorphine (Kelly, Miller & Neumeyer, 1975) compared to sham-operated animals, which may be due to supersensitivity of the denervated mesolimbic DA receptors. LSD (1.0 mg/kg i.p.), like apomorphine (1.0 mg/kg i.p.), produced a marked stimulation of locomotor activity in these animals although this dose did not increase the locomotor activity of control rats. The non-hallucinogen (+)-bromo-lysergic acid diethylamide (2.0 mg/kg i.p.) did not stimulate locomotor activity in 6-OHDA treated rats. The dopamine antagonist pimozide (0.5 mg/kg i.p.) blocked the locomotor stimulation produced by LSD. These results suggest that LSD can act as an agonist at mesolimbic dopamine receptors.

The M.R.C. are thanked for financial support. P.H.K. is an I.C.I. Post-doctoral Research Fellow.

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Biochemical evidence for the involvement of noradrenaline in motor activity produced by L-DOPA in rodents

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The beneficial effects of L-DOPA therapy in Parkinsonian patients have been attributed to the replacement of cerebral dopamine (DA). However, considerable experimental evidence suggests that both noradrenergic and dopaminergic neurotransmission are involved in the control of gross motor activity. We have previously presented behavioural and biochemical evidence to suggest the involvement of a noradrenergic component in the L-DOPA reversal of reserpine-induced akinesia in mice (Dolphin, Jenner & Marsden, 1975). We now extend these studies to include biochemical evidence which suggests that L-DOPA induces functional changes in noradrenaline (NA) turnover. Normal mice ('Swiss s' or 'p' strain male), or those pre-treated with reserpine (10 mg/kg, i.p.) 19 h previously, or α-methylparatyrosine (AMPT) (200 mg/kg, i.p.) 3 h previously, were given L-DOPA (200 mg/kg in methylcellulose i.p.) plus MK486 (α-methyldopahydrazine (25 mg/kg, i.p.)). In all cases a significant increase in whole brain DA was observed 1 h after L-DOPA.

Cerebral NA levels were also significantly increased in reserpinized mice, from 19 ± 6 ng/g to 46 ± 13 ng/g ($P < 0.05$); and in AMPT-treated mice from 249 ± 84 ng/g to 501 ± 108 ng/g ($P < 0.025$).

This increase was prevented by the dopamine-β-hydroxylase inhibitor FLA-63 (bis-(1-methyl 4 homopiperazinyl thiocarbonyl) disulphide) administered 1 h before L-DOPA.

In contrast the same dose of L-DOPA produced no change in NA in normal animals (from 611 ± 43 ng/g to 630 ± 63 ng/g), although pre-treatment with pargyline (200 mg/kg, i.p.) resulted in a significantly greater rise in NA 1 h after L-DOPA (1148 ± 175 ng/g) compared with animals given pargyline alone (832 ± 29 ng/g $P < 0.05$).

Whole brain 4-methoxy 3-hydroxyphenylglycol sulphate (MOPEG-SO_4) measured 2 h after administration of L-DOPA (200 mg/kg) + MK486 (25 mg/kg) was unaltered in normal and AMPT-treated male Wistar rats, but was significantly decreased in reserpinized rats (22 ± 3 ng/g) compared to controls (53 ± 4 ng/g, $P < 0.0125$). However, in agreement with Keller, Bartholini & Pletscher (1974), elevation in whole brain MOPEG-SO_4 was found 2 h after a lower dose of L-DOPA (50 mg/kg, i.p). This was significant in reserpinized rats (90 ± 12 ng/g) compared to controls (53 ± 4 ng/g; $P < 0.025$) and in AMPT-treated rats (125 ± 6 ng/g) compared to controls (75 ± 7 ng/g; $P < 0.0005$). The increase in brain MOPEG-SO_4 after a small dose of L-DOPA was prevented by prior administration of FLA-63.

Measurement of forebrain homovanillic acid (HVA): 3,4-dihydroxyphenylacetic acid (DOPAC) ratios in mice suggested that 2 h after the high dose of L-DOPA the enzyme catechol-*O*-methyl transferase (COMT) was markedly inhibited; the HVA/DOPAC ratio (0.19 ± 0.02) was significantly lower than that found in normal animals (1.01 ± 0.19), or 2 h after the lower dose of L-DOPA (0.87 ± 0.27).

These findings confirm that increased NA turnover does occur after L-DOPA in normal and amine-depleted animals. It cannot be detected by measuring MOPEG-SO_4 levels, after high doses of L-DOPA because these inhibit COMT, thereby preventing the formation of this metabolite of NA.

This work was carried out with the aid of the Medical Research Council and the research funds of the Royal Bethlem and Maudsley hospitals and King's College Hospital Medical School.

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Impaired response control in the rat after 6-hydroxydopamine lesions to the dorsal noradrenaline bundles

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The dorsal noradrenaline (NA) containing pathway in the rat brain arises from the locus coeruleus and innervates the limbic system and cortex. 6-Hydroxydopamine (6-OHDA) 8 μg dissolved in 2 μl of 0.9% sodium chloride solution containing 1 mg/ml of ascorbic acid when injected bilaterally into the dorsal NA bundle at a site anterior to the locus coeruleus results in virtually total and selective loss of NA from the limbic system and cortex.

Ten male albino Wistar rats were given such 6-OHDA lesions and one week later were compared with vehicle treated control animals on the acquisition of a running response for food reward in an L-maze. The 6-OHDA-lesioned animals achieved a stable running speed as quickly as control animals. However, when food was no longer presented in the goal box extinction of this learned running response proceeded more slowly in 6-OHDA-treated rats than in the controls (Trials to extinction, Mann Whitney U-Test $U = 2.0$, $P < 0.001$).

The animals were subsequently trained in a Skinner box to press a lever on a continuous schedule of food reinforcement; the 6-OHDA dorsal bundle lesioned animals were not impaired in learning this new response and after 10 days training were emitting equal numbers of lever presses. Food presentation was then stopped and it was found that during the first two days of extinction, the 6-OHDA-lesioned rats made more responses to the lever ($P < 0.05$) than the controls. Finally, acquisition of a successive visual discrimination in a Skinner box was studied in which the rats, after further training on the continuous food reinforcement schedule, were required to respond for food when the Skinner box houselight was on and to refrain from responding on trials when it was switched off. The dorsal bundle lesioned animals were slow to develop the discrimination habit as they failed to inhibit responses on the non-rewarded dark trials. During the first days of testing the 6-OHDA-lesioned animals consistently recorded more responses during the dark trials (ratio of light to dark lever presses $X^2 = 41.72$ d.f. = 14 $P < 0.001$ and overall responding on dark trials, $X^2 = 35.61$ d.f. = 14, $P < 0.01$).

At the end of the experiments the brains of 5 6-OHDA-treated and 5 control animals were assayed for forebrain noradrenaline and dopamine levels using a radio-enzymatic assay method After the 6-OHDA treatment, noradrenaline levels in the hippocampus were reduced from 0.333 ± 0.069 ng/g to unmeasurable levels and in the cortex from 0.174 ± 0.068 ng/g to 0.001 ± 0.002 ng/g. Dopamine levels in the appropriate forebrain segments were unaffected.

This pattern of impairments in the dorsal bundle animals is characterized by difficulty in extinguishing responses in previously rewarded situations. Rats with surgical lesion to the hippocampus show similar deficits. It is suggested that the classical hippocampal syndrome in the rat can be mimicked to a large degree by selective loss of forebrain NA.

Dopamine and drug-induced hyperactivity in rats

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We have investigated the relations between on the one hand the concentration of dopamine (DA) and its deaminated metabolite dihydroxyphenylacetic acid (DOPAC) in the rat corpus striatum, and on the other the hyperactivity which follows the subcutaneous injection of dexamphetamine sulphate 1.18 mg/kg (DEX), chlordiazepoxide hydrochloride 12.5 mg/kg (CDZP) or a mixture of both these doses (MIX). It had previously been shown that the mixture produced in rats a high level of activity in a Y-maze which could not be elicited by any dose of either drug given separately (Rushton, Steinberg & Tomkiewicz, 1973).

Female hooded rats of about 200 g were killed 20, 40, 60 or 80 min after injection, the striata dissected out and DA and DOPAC levels measured by the methods of Laverty and Sharman (1965) and Murphy, Robinson & Sharman (1969) respectively. At the same times after injection the activity of other groups of rats was tested by placing animals individually in a Y-shaped maze for 5 min, and recording the number of entries into the three arms.

The table shows that DEX raised DA and lowered DOPAC, the peak effects occurring at 40 min, which coincided with peak activity in the Y-maze. Changes in DA closely paralleled changes in activity. CDZP did not significantly alter DA or DOPAC but raised activity in the Y-maze, the peak effects occurring at twenty minutes. The mixture produced at all times more activity than either of the ingredient drugs, and this effect was relatively independent of the time after injection. Changes in DA and DOPAC were however rather similar to those produced by DEX, except that peak effects on DA occurred at sixty minutes.

Since the effects of the three treatments on DA and DOPAC were studied in separate experiments it is not possible to make strict comparisons between them. Nevertheless, the results suggest that the increased activity caused by DEX may be related to an effect on dopaminergic neurones in rat striatum. There is no indication that this is so for CDZP, and the time course of the effect of the mixture on DA and DOPAC bears no obvious relation to the persistently high level of activity induced by the mixture throughout the experiments.

We thank the Medical Research Council for support, and S. Sarsfield for excellent technical assistance.

Table

		Minutes after injection				
		20	*40*	*60*	*80*	*Controls*
DEX	DA	115 ± 5	140 ± 2*	126 ± 7*	126 ± 7*	4.81 ± 0.29 μg/g (4)
	DOPAC	69 ± 9	66 ± 6*	68 ± 11	105 ± 21	1.09 ± 0.13 μg/g (4)
	Entries	143 ± 16*	215 ± 22*	171 ± 26*	192 ± 14*	++
CDZP	DA	111 ± 5	99 ± 5	99 ± 9	104 ± 9	5.41 ± 0.37 μg/g (4)
	DOPAC	97 ± 19	106 ± 10	112 ± 12	106 ± 20	1.20 ± 0.11 μg/g (5)
	Entries	195 ± 32*	179 ± 34*	164 ± 26*	122 ± 10	++
MIX	DA	113 ± 6	121 ± 7	135 ± 5*	124 ± 6*	5.45 ± 0.23 μg/g (4)
	DOPAC	64 ± 8*	60 ± 7*	60 ± 8*	67 ± 2*	1.09 ± 0.12 μg/g (4)
	Entries	251 ± 17*†	246 ± 15*	244 ± 15*†	256 ± 11*†	++

Values are expressed as percentages of control values and are the means ± s.e. mean of at least 5 animals. For DA and DOPAC the calculations are based on the control values from the same experiments only. The number of animals for these controls is shown after the concentration in the controls column.

++ The mean activity for all controls was 15.0 ± 0.7 entries in 5 minutes ($n = 6$).

Student's t tests: * $P < 0.05$ compared with controls.
† $P < 0.05$ compared with DEX

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Do anti-psychotic drugs act by dopamine receptor blockade in the nucleus accumbens?

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There is evidence that central dopamine receptor blockade is responsible for the therapeutic actions of neuroleptic drugs. Two major dopaminergic systems of neurones have been described. One (the nigrostriatal system) shows degeneration in Parkinson's disease, and it has been suggested that extra pyramidal side effects of neuroleptic drugs result from actions on striatal dopamine receptors. A second dopaminergic system terminates in the 'limbic forebrain' region (nucleus accumbens, olfactory tubercle, frontal cortex). Since the anti-psychotic potency of neuroleptic drugs can be dissociated from their extra pyramidal effects it has been suggested that antipsychotic drug actions are related to blockade of 'limbic forebrain' receptors (Bunney & Aghajanian, 1974).

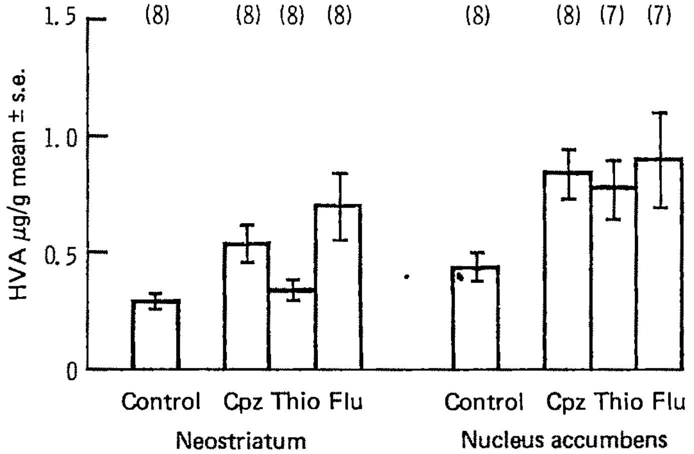

Figure 1 Effects of phenothiazine on HVA concentration in neostriatum and nucleus accumbens. The results show the means ± s.e. mean.
Cpz—Chlorpromazine 5 mg/kg
Thio—Thioridazine 5 mg/kg
Flu—Fluphenazine 0.25 mg/kg

We have attempted to test this hypothesis by studying the effects of drugs with differing ratios of extrapyramidal and therapeutic effects on dopamine turnover in the nucleus accumbens and corpus striatum.

Male Sprague-Dawley rats, weighing 200-220 g were injected with one of the following drugs: chlorpromazine HCl; thioridazine HCl; fluphenazine HCl in dose-ratios approximately equivalent to their therapeutic effects (N.I.M.H., Psychopharmacology Service Center, Collaborative Study Group, 1964). Animals were killed by decapitation, the brains rapidly removed and the striatum and nucleus accumbens dissected out. Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) concentrations were measured fluorimetrically.

The effects of the three drugs on dopamine turnover as assessed by HVA accumulation show quite marked differences in the two brain areas (Figure 1). Whereas the rank order of potencies of the three drugs on dopamine turnover in the corpus striatum is related to their ability to give rise to extra pyramidal side effects, the same doses produce approximately equal effects on dopamine turnover in the nucleus accumbens. The changes appear to be consistent with the hypothesis that anti-psychotic drugs act by blockade of dopamine receptors in the nucleus accumbens. Further experiments have investigated the possibility that differences in drug-induced changes in striatal HVA are due to differences in anti-muscarinic blocking potencies (Miller & Hiley, 1974).

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Tyraminergic mechanisms in rat striatum

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Small concentrations of para-tyramine (*p*-TA) and meta-tyramine (*m*-TA) have been observed in the rat brain (Philips, Durden & Boulton, 1974; Philips, Davis, Durden & Boulton, 1975). Reserpine administration is known to impair the storage mechanisms for neural amines (see review by Shore, 1972) and markedly reduces *p*-TA levels in *Octopus* ganglia (Juorio & Philips, 1975). The accumulated evidence prompted us to investigate the effects of reserpine administration on the concentrations of *p*-TA and *m*-TA in the rat caudate nucleus. For comparison, the effect on the dopamine (DA) concentration was also investigated.

Male Wistar rats of 150-200 g body weight were used throughout. The animals were killed by decapitation and the caudate nuclei quickly dissected out and frozen on dry ice. Tissues from 5 animals were pooled, weighed (approximate weight 300 mg), homogenized and fractions were removed for estimation of the amines. *p*-TA and *m*-TA were estimated as their dansyl derivatives by the mass spectrometric integrated ion current technique through the use of deuterated internal standards (Philips, Durden & Boulton, 1974; Philips, Davis, Durden & Boulton, 1975). DA was estimated fluorimetrically (see Juorio & Philips, 1975).

The concentrations of *p*-TA and *m*-TA in the rat caudate nucleus are respectively 1240 and 4810 times smaller than those of DA (Table 1). All three amines are significantly reduced after subcutaneous administration of reserpine (Table 1); the largest dose (10 mg/kg) reduced concentrations of *p*-TA, *m*-TA and DA by more than 80%, while 6 h after a dose of 0.4 mg/kg, the levels of the three amines were reduced by half (Table 1). These results strongly suggest that striatal tyramines are stored by a reserpine-sensitive mechanism. Recent experiments have shown that the turnover of tyramines in neural tissues is very fast (Wu & Boulton, 1974; Juorio & Philips, 1975; Boulton, Juorio, Philips & Wu, 1975) and that *p*-TA is associated with a brain synaptosomal fraction (Boulton & Baker, 1975). The present findings are further evidence that

Table 1 Effect of reserpine on the concentration of *p*-tyramine (*p*-TA), *m*-tyramine (*m*-TA) and dopamine (DA) in the rat caudate nucleus.

Dose *mg/kg*	*Time after injection* *h*	*p-TA* *ng/g*	*m-TA* *ng/g*	*DA* *ng/g*
–	–	10.1 ± 0.9 (13)	2.6 ± 0.2 (11)	12500 ± 737 (13)
0.2	24	11.9 ± 0.9 (3)	2.5 ± 0.2 (3)	10067 ± 263 (3)**
0.4	6	5.0 ± 0.5 (4)***	1.4 ± 0.06 (4)***	6540 ± 417 (4)***
0.4	12	10.6 ± 0.4 (4)	1.8 ± 0.1 (4)**	8444 ± 219 (4)***
0.4	24	8.3 ± 0.6 (6)	2.0 ± 0.1 (6)*	7273 ± 441 (6)***
0.4	96	8.8 ± 0.8 (4)	2.0 ± 0.1 (4)*	8107 ± 231 (4)***
1.0	24	1.5 ± 0.4 (8)***	0.47 ± 0.2 (9)***	1444 ± 90 (9)***
10.0	24	0.6 ± 0.3 (4)***	0.40 ± 0.11 (4)***	490 ± 40 (8)***

Reserpine was administered subcutaneously. Results are given in ng/g fresh tissue (± s.e. mean) and corrected for recoveries. Student's *t*-test. $^{*}P < 0.025$, $^{**}P < 0.01$, $^{***}P < 0.001$.

tyramines may have a function of their own in neuronal transmission

We thank the Psychiatric Services Branch, Province of Saskatchewan and the Medical Research Council of Canada for financial support.

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Pre- and postsynaptic actions of neuroleptic drugs

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In the present studies neuroleptic drugs of various chemical classes were compared as inhibitors of the postsynaptic dopamine-sensitive adenylate cyclase in rat striatum (Miller, Horn & Iversen, 1974), and for their ability to influence a variety of presynaptic mechanisms in dopaminergic nerve terminals of this brain area (Seeman & Lee, 1975). Sixteen chemical analogues of the butyrophenone haloperidol were tested as inhibitors of the dopamine-sensitive adenylate cyclase, using a fixed concentration of 100 μM dopamine. The drugs inhibited the dopamine response with IC_{50} values ranging from 1 μM to greater than 100 μM, and the results showed a good correlation between dopamine antagonist potencies in this system and the known *in vivo* effects of the same drugs as apomorphine antagonists in the dog (data from Drs P. Laduron & P. Janssen). In intact synaptosome preparations from rat striatum apomorphine was a potent inhibitor ($IC50 = 0.2\ \mu M$) of the conversion of tritiated L-tyrosine to catechols. Other dopamine-mimetic drugs (epinine, dopamine and 2-amino-6, 7-dihydroxy-1,2,3,4, tetrahydronaphthalene, ADTN) had similar inhibitory effects on tyrosine hydroxylation, and were approximately equipotent with apomorphine. Noradrenaline was also effective but less potent than the other compounds, and at high concentrations (10 μM) phenylephrine and isoprenaline also had some inhibitory actions. The effects of dopamine, noradrenaline, epinine and ADTN were significantly reduced in the presence of the dopamine uptake inhibitor benztropine (2 μM), suggesting that they act at least in part by inhibition of intra-synaptosomal tyrosine hydroxylase after uptake into dopaminergic synaptosomes. The actions of apomorphine, however, were unaffected by benztropine, suggesting a direct action on presynaptic 'autoreceptors' at dopaminergic terminals. All of the compounds were at least 50 times less potent as inhibitors of free tyrosine hydroxylase in detergent-containing striatal homogenates. The inhibitory effects of apomorphine on tyrosine hydroxylation in intact synaptosomes were partially reversed by various neuroleptic drugs (see also Christiansen & Squires, 1974), and this appeared to be due to a competitive antagonism between these drugs and apomorphine at presynaptic receptor sites. The neuroleptics themselves, however, also tended to inhibit tyrosine hydroxylation when added alone. Haloperidol, spiroperidol and pimozide were particularly potent in reversing the presynaptic actions of apomorphine on tyrosine hydroxylation, being active at concentrations of less than 10^{-7} M. Neuroleptic drugs had some actions as inhibitors of ^{3}H-dopamine uptake and as dopamine releasers in striatal synaptosomes. They also antagonized the release of ^{3}H-dopamine evoked by protoveratrine. None of these effects, however, occurred at very low drug concentrations and the butyrophenones were no more potent than chlorpromazine. It is concluded that neuroleptics possess actions on both pre- and postsynaptic sites in the striatum, but that the postsynaptic actions are most likely to be crucial in determining the clinical activity of these drugs.

R.J.M. is an M.R.C. Scholar.

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Monoamine oxidase activity in distinct populations of rat brain mitochondria

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Lai, Walsh, Dennis & Clark (1975) separated three distinct populations of mitochondria from rat brain by discontinuous Ficoll gradient. The mitochondria were metabolically active and relatively free of non-mitrochondrial material Two of the mitochondrial populations (SM and SM2) were derived from synaptosomes and the remainder were 'free' mitochondria (M).

Johnston (1968) described two forms of monoamine-oxidase (MAO) in rat brain—type A and type B—on the basis of inhibition studies. Later work has shown that serotonin (5-HT) and phenylethylamine are preferentially deaminated by type A and type B MAO respectively, whereas tyramine is a substrate for both types of the enzyme. Type A MAO is relatively sensitive to clorgyline and type B to deprenyl.

Three populations of mitochondria were prepared by a modification of the procedure of Lai, Walsh, Dennis & Clark (1975). The three populations exhibited distinctly different MAO activities when assayed with 5-HT and phenylethylamine as substrates in a radiometric technique similar to that described by Robinson, Lovenberg, Keiser & Sjoerdsma (1968).

Table 1 MAO activity in mitochondrial populations

n	*Mitochondria*	*5-HT*	*Phenylethylamine*
8	M	51.6 ± 16,4	15.6 ± 7.5
8	SM	117.5 ± 16.6	31.7 ± 5.8
8	SM2	106.6 ± 3.0	51.8 ± 4.6

Results (mean ± s.d.) expressed as nanomoles product formed/mg protein per 30 min (analysis of a contingency table gave $\chi^2 = 48$ $P > 0.0001$).

Inhibition studies with clorgyline and deprenyl revealed that MAO of the lighter synaptosomal mitochondria (SM) was considerably more sensitive to inhibition by clorgyline than the M or SM2 mitochondria or a crude mitochondrial preparation. SM mitochondrial MAO was inhibited approximately 50% by 10^{-12} M clorgyline. The same population of mitochondria (SM) was the least sensitive to deprenyl although the differences in inhibition of MAO of the three sets of mitochondria were less pronounced with deprenyl than with clorgyline. When percentage inhibition of MAO activity was plotted against concentration of clorgyline or deprenyl double sigmoid curves resulted for all three mitochondrial populations with the plateaus suggesting A B MAO ratios of about 4·1 for SM and 1.5.1 for M and SM2.

In agreement with previous findings (Youdim & Sourkes, 1965) heat inactivation at 50°C for 1 h resulted in the loss of about 15% of MAO activity of a crude mitochondrial preparation when tyramine was used as substrate. In contrast the purified mitochondria M, SM and SM2 lost approximately 75% of enzyme activity when similarly treated.

Attempts to fractionate further the SM mitochondria will be discussed.

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Noradrenaline-sensitive adenylate cyclase in rat limbic forebrain homogenates: effects of agonists and antagonists

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Recently Chasin, Mamrak & Samaniego (1974) have demonstrated an adenylate cyclase system in cell-free homogenates of guinea-pig brain that is sensitive to low concentrations of noradrenaline and adrenaline. Blumberg, Taylor & Sulser (1975) have shown that an adenylate cyclase in rat limbic forebrain slices can be specifically stimulated by low concentrations of noradrenaline ($EC_{50} = 2.7 \times 10^{-6}$ M) whilst dopamine is inactive. It is also known that various neuroleptics antagonize this effect (Blumberg & Sulser, 1974; Blumberg *et al.*, 1975). We now report the pharmacological properties of a noradrenaline sensitive adenylate cyclase in homogenates of the rat limbic forebrain. Slices of rat forebrain were prepared by making two coronal cuts, the first being at the optic chiasm, and the second 3 mm further rostral. From this slice the corpus striatum and the adjacent cortex were removed. The tissue homogenization and incubation conditions were essentially those of Chasin *et al.* (1974), in the initial preincubation adenine (1.0 μM) was added. Cyclic AMP concentrations were estimated by the method of Brown, Ekins & Albano (1972). The concentration of 1-NA required for half-maximal stimulation (EC_{50}) was 8.5×10^{-7} M (Table) and

Cyclic AMP production in rat limbic forebrain homogenates

Agonists	*Max. stimulation %*	*EC_{50}(M)*
(—)-Isoprenaline	100	2.0×10^{-7}
(—)-Noradrenaline	100	8.5×10^{-7}
(—)-Adrenaline	100	1.0×10^{-6}
(±)-dl-α-Methylnoradrenaline	100	1.5×10^{-6}
(+)-d-Noradrenaline	25.0 ± 4 9 (4) (0.1 mM)	
(+)-d-Adrenaline	67 0 ± 11.6 (4)	4.5×10^{-6}

Inactive up to 10^{-3} M: Dopamine, (+)-isoprenaline, (±)-octopamine, (±)-normetanephrine, carbuterol, salbutamol

Antagonists	*IC_{50}(M)*	*Antagonists*	*% Inhibition*
(—)-Propranolol	7.0×10^{-7}	(±) Practolol	45.2 ± 2.7 (4) (10^{-4}M)
(±)-Propranolol	8.3×10^{-7}	(±) Dichloroisoprenaline	45.7 ± 7.2 (4) (10^{-4}M)
(+)-Propranolol	8.4×10^{-5}	(±) MJ 1999	51.5 ± 4.1 (4) (10^{-4}M)
(—)-Alprenolol	5.0×10^{-6}	Phentolamine	44.05 ± 3.3 (7) (10^{-5}M)
(±)-Alprenolol	5.7×10^{-6}		
(+)-Alprenolol	2.0×10^{-5}		

Inactive at 10^{-4}M: H35/25, (±)-INPEA

Neuroleptics	*% Inhibition at 10^{-5}M*	*Neuroleptics*	*% Inhibition at 10^{-5}M*
Promazine	83.5 ± 4.8 (7)	α-Chlorprothixene	48.9 ± 14.4 (4)
(+)-Butaclamol	73.9 ± 10.4 (3)	β-Clopenthixol	44.9 ± 2.8 (4)
Clozapine	66.2 ± 2.9 (4)	Pimozide	44.6 ± 2.8 (5)
β-Chlorprothixene	57.4 ± 13.3 (4)	Haloperidol	43.5 ± 3.0 (4)
Thioridazine	55.0 ± 5.1 (3)	Trifluperazine	39.6 ± 6.6 (4)
Chlorpromazine	50.9 ± 0.9 (4)		
	Ki ≃ 1.6×10^{-7}M	α-Flupenthixol	38.6 ± 2.6 (3)
α-Clopenthixol	48.9 ± 7.0 (4)	β-Flupenthixol	33.9 ± 5.9 (4)
		(—)-Butaclamol	23.4 ± 3.0 (4)

Inactive at 10^{-5}M: Desmethylimipramine, 7-hydroxy-chlorpromazine, chlorpromazine sulphoxide

EC_{50}. Concentration required for half-maximal stimulation by the agonist. IC_{50}· Concentration of antagonist required to inhibit by 50% the stimulation produced by 50 μM (—)-NA. Ki· Inhibition constant. In experiments using antagonists or neuroleptics the drug was added 15 min prior to the addition of 50 μM (—)-NA. Figures in brackets represent the number of determinations. EC_{50} and IC_{50} values were estimated graphically from the means of at least four determinations.

maximum stimulation occurred over the range $1 \times 10^{-5} - 5 \times 10^{-5}$M. A 2.5 fold increase in cyclic AMP levels was routinely obtained. The pharmacological characteristics of agonists on this system are summarized in the table. The structures of active and inactive molecules demonstrates the strict requirements for a catechol grouping and a β-hydroxyl group with the correct stereochemistry. The blockade of the stimulation by 5×10^{-5}M noradrenaline in the presence of adrenolytics was stereoselective. The selective β_1 antagonist (±)-practolol (Dunlop & Shanks, 1968) was less active than (±)-propranolol, whilst the γ blocker H35/25 (1-(4′-methylphenyl)-2-isopropylamino-propranol HCl) (Levy & Wilkenfeld, 1969) was inactive; this is of interest as the β_2 agonist salbutamol (Farmer *et al.*, 1970) was also found to be inactive. The spectrum of activity for the neuroleptics was very different from that found for the blockade of the dopamine sensitive adenylate cyclase (Miller, Horn & Iversen, 1974). Promazine, clozapine, thioridazine and chlorpromazine were all quite active, whereas α-flupenthixol and trifluperazine, both potent blockers of the dopamine sensitive adenylate cyclase, were less active. Assuming the blockade by chlorpromazine is competitive it has an approximate Ki of 1.6×10^{-7}M whereas the value for the inhibition of the dopamine-adenylate cyclase is 4.8×10^{-8}M (Miller *et al.*, 1974). It is also of interest that no large differences in potency were found for the thioxanthene isomers; this contrasts strongly with results obtained from the dopaminergic cyclase (Miller *et al.*, 1974).

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Altered sensitivity of β-adrenoceptor-mediated cyclic AMP formation in brain

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In several studies using homogenates or slices of brain tissue it has been demonstrated that the stimulation of catecholaminergic receptors causes an increased synthesis of cyclic AMP (Daly, 1975). In our studies of the effects of neurohormones on cerebral cyclic AMP metabolism we have found that the neonate chick with its immature blood-brain barrier is a useful experimental model for both *in vivo* and *in vitro* investigations (Edwards, Nahorski & Rogers, 1974), and we have provided evidence that catecholamines stimulate formation of the nucleotide in chick cerebral hemispheres via β-adrenoceptors (Nahorski, Rogers & Smith, 1974). In order to assess the potential importance of these responses we have, in the present study, used procedures that alter central adrenergic transmission, in an attempt to determine whether or not there are accompanying changes in the sensitivity of the cyclic AMP response.

Experiments were performed on 1-6 day old male Ranger chicks. Pretreatment with reserpine (2.5 mg/kg s.c.) daily for three days or 6-hydroxydopamine (60 μg in 10 μl intracerebroventricularly) on two successive days severely depleted brain catecholamines (> 70%) for at least six days. Five days after commencing the pretreatments, groups of chicks were injected intravenously with (−)-isoprenaline (5 μmol/kg) or histamine

(100 μmol/kg). The cerebral hemispheres were removed by freeze-blowing 2 min later and cerebral cyclic AMP measurements were performed by a protein binding assay. The increase in cyclic AMP in response to isoprenaline in reserpinized chicks (475%) or in chicks pretreated with 6-hydroxydopamine (441%) was significantly greater than that produced in control birds (266%), indicating an increased sensitivity to isoprenaline following the chronic depletion of cerebral catecholamines. Experiments were performed therefore to determine whether the chronic administration of isoprenaline would conversely reduce the sensitivity of the cyclic AMP response. (−)-Isoprenaline was suspended in glycerol trioleate and administered subcutaneously (150 μmol/kg) in two doses at 12 h intervals to effect a slow release of catecholamine. Six hours after the second injection the cerebral cyclic AMP response to intravenous isoprenaline was almost completely suppressed. The increase in cyclic AMP induced by histamine *in vivo* was not influenced by any of the drug treatments.

More detailed studies were performed *in vitro* using slices of chick cerebral hemispheres. Dose-response curves for isoprenaline indicated that the maximal increase in cyclic AMP produced by the catecholamines was significantly enhanced in slices prepared from reserpinized chicks but severely suppressed in the chronic isoprenaline group. However, although the supersensitive response was still observed in the presence of the potent phosphodiesterase inhibitor, Ro 20-1724 (4-(3-butoxy-4-methoxy benzyl)-2-imidazolidinone) (200 μM) the subsensitive response was almost restored to that of the controls.

The data suggest that the sensitivity of cerebral β-adrenoceptors mediating cyclic AMP formation may be regulated by the functional amount of transmitter at the receptor. The subsensitive condition may, in part, be due to a selective increase in phosphodiesterase activity but several alternative mechanisms need to be examined.

This work was supported by a grant from the Wellcome Trust.

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Comparison of the effects of DOPA and noradrenaline on single cortical neurones

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L-3, 4-dihydroxyphenylalanine (DOPA) is widely used in the treatment of Parkinson's disease. DOPA is a metabolic precursor of dopamine, and it is generally believed that DOPA exerts its therapeutic effect via the release of dopamine in the caudate nucleus (Hornykiewicz, 1974). However, DOPA is also known to be a precursor of noradrenaline (NA), and it has been suggested that in structures receiving a NA innervation, exogenously administered DOPA may cause the release of NA from pre-synaptic terminals and thus mimic the actions of NA on post-synaptic cells (Andén, Carlsson & Häggendal, 1969). We have used the technique of microelectrophoresis to compare the action of DOPA and NA on single neurones in the cerebral cortex, a structure rich in NA-containing terminals (Fuxe, 1965).

Single spontaneously-active cortical neurones were studied in cats and rats anaesthetized with halothane. Drugs were applied from six-barrelled micropipettes by microelectrophoresis. Our techniques and methods of study have been described elsewhere (Bevan, Bradshaw, Roberts & Szabadi, 1974).

The effect of DOPA (released from 0.05 M DOPA methyl ester HCl solution) was examined on 51 neurones in the rat; 44 were excited and 7 depressed by the drug. Eleven cortical neurones were studied in the cat; of these 10 were excited and one depressed by DOPA. When the effects of DOPA were compared with those of NA on the same cells it was found that neurones invariably responded in the same direction to the two drugs (40 cells). In the case of both excitatory and depressant effects DOPA appeared to be less potent than NA, both in terms of the intensity of the ejecting current required to evoke equivalent responses, and in terms of the magnitude of the responses evoked by identical ejecting pulses. However, this apparent difference in potency might be due to a difference between the physical mobilities of the two drugs.

Excitatory responses to both DOPA and NA could be reversibly antagonized by phentolamine (12 cells). Excitatory responses to DOPA and NA could also be antagonized by propranolol (6 cells); responses to acetylcholine were not affected. On the other hand, excitatory and depressant responses to DOPA were not affected by atropine applied with ejecting currents sufficient to antagonize excitatory responses to acetylcholine (6 cells).

These results show that cortical neurones are sensitive to locally administered DOPA, and suggest that DOPA may activate receptors similar to those activated by NA. However, it is not clear from the present results whether DOPA acts directly on post-synaptic receptors, or indirectly via the release of NA from pre-synaptic terminals.

This work was supported by the Mental Health Trust and Research Fund.

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Induced receptors for noradrenaline and serotonin in guinea-pig vas deferens?

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We have previously reported recovery of responsiveness to noradrenaline during exposure to methacholine in tissues whose α-adrenoceptors were completely blocked by phenoxybenzamine (Iijima & Reiffenstein, 1972). Methacholine could also induce serotonin receptors. Further investigation of these induced receptors is reported here. Stripped guinea-pig vas deferens was mounted in Krebs solution at 37°C and isotonic contractions obtained under 0.5 g tension. Agonist concentrations used throughout were maximal. Induced serotonin receptors were investigated in normal tissues, while induced α-adrenoceptors were studied in tissues pretreated with phenoxybenzamine until response to noradrenaline was eliminated. Contractions were then obtained to serotonin, or noradrenaline, in the presence of a receptor-inducing agent (methacholine and others). To investigate whether noradrenaline and serotonin might be reacting with the same receptor, comparative blocking studies were done by cumulative additions of antagonists during responses to combined inducer (methacholine) and agonist. The induced α-adrenoceptors were as susceptible to phentolamine ($ID_{50} = 1.2 \times 10^{-5}$M *vs.* noradrenaline) as were normal α-adrenoceptors ($ID_{50} = 2 \times 10^{-5}$ M). Methysergide (up to 10^{-3} M) failed to inhibit induced responses to noradrenaline. The induced response to serotonin could be blocked by phentolamine ($ID_{50} = 1.8 \times 10^{-4}$ M) but lower concentrations of methysergide were effective ($ID_{50} = 3 \times 10^{-5}$ M). Thus it is likely that two distinct receptors were involved. Agents other than methacholine also proved capable of reviving responsiveness to noradrenaline: KCl (1×10^{-2} M), $BaCl_2$ (2×10^{-8} M), or propranolol (2×10^{-4} M). The latter has been observed to have a similar effect in vasculature (Yamamura & Horita, 1968; Janis & Triggle, 1974). By themselves, these agents produced widely different contractions (or none at all) so that contraction does not seem to be a requirement. Should the substances, such as methacholine or propranolol, producing this effect depolarize the smooth muscle and thereby reduce the threshold for spike generation, then previously subthreshold receptor activation could then produce contraction and there would be no need to postulate the induction of new receptors. The revived responses to noradrenaline were also potentiated by cocaine, but cocaine had no effect in the absence of the receptor-inducing agent (when noradrenaline itself produced no effect). This supports the contention of Nakatsu & Reiffenstein (1968) that cocaine increases maximal responses to noradrenaline by increasing the efficacy of unblocked receptors rather than uncovering phenoxybenzamine-inhibited receptors. We also confirmed our original observation that the induced α-adrenoceptors were insensitive to phenoxybenzamine. The tissues were re-exposed to phenoxybenzamine in the presence of methacholine (which would be expected to protect the muscarinic receptors, and maximally induce α-adrenoceptors). Responses to noradrenaline were only reduced in proportion to the responses to methacholine. This suggests that methacholine is temporarily uncovering or unblocking the phenoxybenzamine-inhibited α-adrenoceptors. A corollary is that phenoxybenzamine does not alkylate directly to the α-adrenoceptor. This phenomenon could not be duplicated in guinea-pigs from the U.K. since their vasa deferentia do not contract in response to noradrenaline.

Supported by Medical Research Council of Canada (MA 4979).

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The effect of chlorpromazine on carbachol binding to muscarinic receptors in intestinal smooth muscle

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The binding of agonists to muscarinic receptors in strips of longitudinal muscle from guinea-pig small intestine, as measured by the inhibition of the binding of an affinity label for the muscarinic receptor, [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM), differs from that of antagonists. Whereas antagonist binding curves in general approximate well to those expected for a simple mass-action interaction, those for agonists show an apparent negative co-operativity (Young, 1974). Similar effects have been observed using a microsomal fraction (Burgen & Hiley, 1975). There is other evidence that the binding of agonists differs from that of antagonists. Exposure of muscle strips to distilled water prior to the usual 1 h preincubation in normal Krebs solution results in a shift of the binding curve for carbachol to the left without any apparent change of slope, whereas the curve for methylatropinium bromide is unaltered (Taylor & Young, unpublished observations). What structural element is modified by the hypotonic treatment leading to the long-lasting changes in carbachol binding, and in particular whether this is an effect on the receptor macromolecule or its supporting membrane, is unknown. In an attempt to explore the relationship between membrane structure and receptor conformation we have examined the effect of chlorpromazine, a drug whose membrane actions have been extensively studied (Seeman, 1972).

Chlorpromazine, added 30 min prior to the mustard and present throughout, acted as a competitive inhibitor of [^{3}H]-PrBCM binding ($K_a = 1.4 \times 10^6\ \mathrm{M}^{-1}$), in agreement with Miller & Hiley (1974). However, treatment of the muscle strips for 30 min at room temperature (22°) with higher concentrations of chlorpromazine ($10^{-5} - 10^{-4}$ M), corresponding to the prelyticlytic range on erythrocytes, resulted in a decrease in [^{3}H]-PrBCM binding measured after 60 min washing in Krebs solution alone. Atropine (10^{-6} M) did not prevent this long-lasting effect of chlorpromazine. The loss of binding was apparently not due to solubilization of receptor material since 10^{-4} M chlorpromazine failed to remove the tritium from strips previously labelled with [^{3}H]-PrBCM. The curve for the inhibition by methylatropinium bromide of [^{3}H]-PrBCM binding to strips pretreated with 2×10^{-5}M chlorpromazine was unaltered from that to normal strips (best fit parameters: Hill slope, 0.99; K_a, $1.2 \times 10^8\ \mathrm{M}^{-1}$; non-specific binding of [^{3}H]-PrBCM, 13%). In contrast the mean Hill slope of the binding curve deduced for carbachol was increased from 0.4 in normal strips to 0.6 in chlorpromazine pretreated strips, with a small increase in the ED_{50}. Thus pretreatment of longitudinal muscle strips with prelytic concentrations of chlorpromazine has an effect on the binding curve for carbachol similar to that of prior desensitization.

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Is the latency of parasympathetic nerve effects due to a slow activation of muscarinic receptors? An iontophoretic study.

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The latency between parasympathetic nerve stimulation and the response of the smooth muscle membrane is in the range 0.07-0.4 s (Gillespie, 1962; M.R. Bennett, 1966; Ohashi & Ohga, 1967; T. Bennett, 1969; Furness, 1969; Ito & Kuriyama, 1971). A possible explanation of this latency is that it is caused by a combination of slow conduction and synaptic delays in the nerve pathways by which excitation reaches the muscle membrane. If this was the case then one would expect the response to iontophoretically applied acetylcholine or carbachol to have a latency much shorter than 0.1 second.

Experiments were therefore done in which carbachol was applied from iontophoretic pipettes (resistance about 200 MΩ) to the surface of smooth muscle from guinea-pig ileum and taenia in isotonic or in sucrose hypertonic physiological salt solution. Simultaneously intracellular records were made from the smooth muscle by microelectrode which was inserted as close as possible to the tip of the carbachol pipette. In most experiments the distance between electrode and pipette was proably not more than 20 μm.

In isotonic solution spikes were often discharged spontaneously. When carbachol was applied iontophoretically no latencies less than about 0.1-0.2 s were observed. The depolarization produced by carbachol invariably triggered a spike or spikes and the electrode was usually dislodged by the contraction of the muscle.

In sucrose hypertonic solution the muscle does not contract and the membrane potential is stable. The sensitivity to carbachol (either applied in the perfusate or iontophoretically) was reduced. Iôntophoretic application of carbachol produced small depolarizations of up to 10 mV. Their latency was again never less than 0.1-0.2 seconds.

In these experiments carbachol was released onto the surface of the muscle strip. Presumably therefore it must first cross a layer of serosal cells before it can affect the muscle. By 0.1 s after beginning release of carbachol, it can be calculated that a patch of muscle surface some 100 μm in diameter must be exposed to a suprathreshold concentration of carbachol in these experiments. The observed latency could be explained by invoking some special property of the serosa or associated connective tissue. However, Purves (1974) observed similar latencies when acetylcholine was applied iontophoretically to cultured taenia smooth muscle cells where presumably serosa is absent. A simple explanation of the latencies observed in his experiments and in mine, or following parasympathetic nerve stimulation, is that it represents mainly the time required to produce the responses associated with activation of the muscarinic receptor.

This was done during the tenure of a Royal Society Locke Research Fellowship and was supported by the MRC.

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DEMONSTRATIONS

Changes in the MAO activity with age in the rat vas deferens

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The activity of monoamine oxidase (MAO) in many animal tissues alters with age and hormones. In the rat heart the changes in activity are influenced by the particular substrate used (Lyles & Callingham, 1974). This may be explained by the presence of at least two possible forms of MAO, called 'species A' and 'species B' (Johnston, 1968).

In the rat vas deferens, use of the irreversible MAO inhibitor clorgyline, has shown that 5-HT is metabolized by species A, benzylamine by species B and tyramine by a mixture of A and B (Jarrott, 1971). In this tissue it has been reported that the MAO activity does not alter with age (Sampath & Clarke, 1972). However, tryptamine was used as the only substrate. Three other substrates, 5-HT, benzylamine and tyramine, have now been used to determine whether any changes in MAO activity with age can be detected or not and to see if any such change could be accounted for by alteration in the proportions of species A and B.

MAO was assayed radiochemically using [^{3}H]-5-HT, [^{3}H]-tyramine and [^{14}C]-benzylamine, and expressed as nmol substrate consumed (mg protein)$^{-1}$ h^{-1}. Male Wistar rats were used in groups with body weights of about 40, 60, 110, 160, 210, 300 and 500 g. The vasa deferentia from two rats were homogenized in 1 mM potassium phosphate buffer, pH 7.8. The values represent the mean of 3-4 of these pooled homogenates in each body weight group (i.e. n = 6-8 animals).

Total vas deferens weight was found to increase steadily throughout the growth of the rat. The specific activity of MAO with all three substrates was found to increase between 40-60 g, and then to decline with age. However, the rate of this decline depended upon the substrate used. It was most rapid at the early age (60-160 g) using benzylamine, slowest with 5-HT and intermediate with tyramine. Conversely, at older ages (160-500 g) the rate of decline was faster with 5-HT than with benzylamine, with tyramine again being intermediate in rate.

MAO activity was measured in the presence of clorgyline concentrations of 5×10^{-11} to 5×10^{-4} M, for each of the various age groups. At all ages studied, 5-HT and benzylamine gave inhibition curves confirming their metabolism by enzyme A and B respectively. Tyramine gave a biphasic inhibition curve with a plateau region; the position of this plateau varies with age, suggesting variable proportions of enzyme A and B. Proportions of enzyme A at different body weights were: 60 g-65%, 110 g-73%, 160 g-78%, 500 g-57%.

From these results it would appear that the MAO activity measured with all three substrates, after reaching a peak, declines with age. However, the rate of decline is determined by the particular substrate and it also corresponds with the changes that occur with age in the proportions of species A and B MAO in the rat vas deferens.

Supported by a grant from the British Heart Foundation. G.A.L. is a Medical Research Council Scholar.

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Radiochemical assay of catecholamines in blood plasma

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At present, the most promising approach to the assay of the very small quantities of the three catecholamines, known to be present in normal blood plasma, is the use of a suitable enzyme that allows the formation of identifiable radioactively labelled products. Passon & Peuler (1973) used the method of Engelman, Portnoy & Lovenberg (1968), in which a preparation of the enzyme catechol-O-methyl transferase enables the introduction of a radioactively labelled methyl group into the catecholamine molecule, and were able to carry out the reaction directly in small volumes of blood plasma Fry, House & Sharman (1974) estimated the content of the catecholamines in the salivary gland of the cockroach after separation of such radioactive compounds, as their acetylated derivatives, by paper chromatography This method of paper chromatographic separation has been preferred in the assay to be demonstrated since it allows the complete separation of acetylated, radioactive metanephrine, normetanephrine and methoxytyramine to be made with little difficulty. The acetylation of the methoxy-amines increases their stability so that there is little or no loss during chromatographic separation. We feel that there are fewer hazards in handling the paper chromatograms than with other methods of separation since the radioactive regions are simply cut out and eluted in the scintillation vial.

The demonstration will show the method used for the simultaneous estimation of noradrenaline, adrenaline and dopamine in the same sample of blood plasma and in small pieces of tissue. The reproduceability and sensitivity of the method will be illustrated and some results which have been obtained in studying widely different problems concerning catecholamines will be presented to demonstrate the suitability of the method for the screening of clinical material.

We thank the East Anglia Regional Health Authority for their generous support (Local Research Grant No. 64). J.P.F. is supported by an A.R.C. Research Studentship.

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A method for the micro-injection into mammalian muscle fibres of procion brilliant red H3BN

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A variety of techniques for the preparation and use of micropipettes filled with Procion dyes have been described (see Kater & Nicholson, 1973). The present method is simple and deviates little from the method used in our laboratories for the preparation of conventional recording microelectrodes.

Procion Brilliant Red H3BN, when obtained from source (I.C.I. Limited, Dyestuffs Division), is contaminated with a water insoluble de-dusting agent (Stead, 1973). This agent was first removed by rinsing with acetone. A solution of the dye (4% w/v) was then prepared by dissolving this partially purified product in distilled de-ionized water and filtering the solution through a 0.45 µm millipore filter. Micropipettes were pulled from 2 mm bore pyrex glass tubing. They were filled by boiling under reduced pressure with industrial methylated spirit or absolute ethanol, transferred to distilled water for 5 minutes, and then immersed overnight in a dye solution. Pipettes thus filled with Procion Brilliant Red had tip resistances of 20-30 MΩ and tip potentials less than 10 mV. Conventional microelectrodes filled with 3M KCl prepared by a similar procedure had tip resistances of 5-15 MΩ and tip potentials less than 10 mV. Dye-filled pipettes prepared in this manner and stored in Procion Brilliant Red solution would usually pass current and eject dye up to two weeks after their manufacture but fresh solutions and electrodes were generally prepared once per week. Pipettes filled with the dye were capable of passing direct currents and square wave pulses of either polarity to magnitudes greater than 200 nA. Dye was ejected by anodal current. When a pipette was inserted into a muscle fibre a suitable rate of dye injection was effected by currents of 20-80 nA Direct currents of 40 nA were normally used. Larger currents often caused blockage of the pipette due to coagulation of dye at the tip. A noticeable 'mark' could be seen in the muscle fibre within twenty seconds. Rapid fixation was necessary since the dye appears to diffuse from the point of injection within 5-10 minutes For histological examination, cold Calcium Formol fixation (4°C for 4 h) results in low background fluorescence and the marked fibre is easily detected under a Fluorescence microscope (excitation 380 nm, emission 540 nm). For electron microscopic examination, the usual glutaraldehyde/osmium fixative is unsuitable as the former induces intense background fluorescence and the latter quenches all fluorescence completely Of other fixatives tested, one comprising 0 8% glutaraldehyde 5% paraformaldehyde buffered to pH 7.2 has been used with some success.

This work was supported by grants from the Muscular Dystrophy Associations of America Inc. and the Muscular Dystrophy Group of Great Britain R R.R. is in receipt of an M.R.C. Studentship for training in Research Methods.

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The use of halothane-induced sleeping time as an index of central nervous system excitability

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Sleeping time following an i.p. injection of a central nervous system (CNS) depressant drug e.g. hexobarbitone or pentobarbitone has often been used as an index of CNS excitability, particularly in drug tolerance studies. However, we have reported previously that a change in the rate of drug metabolism tends to mask any change in sensitivity of the CNS (Stevenson & Turnbull, 1970) and more useful information on the sensitivity of the brain to barbiturate may be obtained by determining the duration of anaesthesia following an intracerebroventricular injection of pentobarbitone (Stevenson & Turnbull, 1974). However, this latter method is unsuited to the study of change in CNS sensitivity occurring with time. We have therefore explored the usefulness of the measurement of the duration of anaesthesia produced by carefully controlled exposure to halothane.

The gas flow from an anaesthetic apparatus, incorporating a Fluotec Mark 2 vaporizer, was passed through a perspex box of dimensions 35 x 15 x 10 cm in which up to six 200 g rats could be placed. Oxygen containing 5% halothane was passed through the box at a flow rate of 2 litres min^{-1} for 3 min and the rats were exposed to this mixture for a further 5 min after switching off the gas flow. The animals were then removed from the box, placed on cotton wool and the time taken to regain the righting reflex was determined for each animal using a stopwatch A sleeping time of between 3 min and 7 min, depending on the time of day at which animals were exposed to halothane, was obtained.

Pretreatment of animals with a CNS stimulant (amphetamine 1 mg kg^{-1} for 10 min) or depressant (sodium pentobarbitone 5 mg kg^{-1} for 5 min) shortened (2.58 ±0.18 (3) (± s.e.m.) min) or lengthened (9.18 ± 0.10 (3) min) the sleeping time respectively compared with saline pretreated rats (6.85 ± 0.17 (4) minutes).

Induction or inhibition of hepatic drug-metabolizing enzyme activity had no effect on the halothane-induced sleeping time (phenobarbitone pretreated 5.38 ± 0.20 (4); SKF 525A (2-diethylaminoethyl-2,2-diphenylvalerate HCl) pretreated 5.67 ± 0.26 (4); control 5.25 ± 0.10 (5) minutes).

Halothane-induced sleeping time could be repeatedly determined in the same animals with no evidence of tolerance, although a marked diurnal variation was observed. We have made repeated halothane sleeping time determinations in rats pretreated both acutely and chronically with a number of CNS depressant drugs and have found that drug exposure is followed by a cross-tolerance to halothane. This tolerance is frequently followed by a post-tolerance hypersensitivity to halothane and brain halothane concentrations determined on awakening confirm that the tolerance and hypersensitivity are due to changes in the sensitivity of the CNS to the anaesthetic.

Thus it is concluded that determination of halothane-induced sleeping time appears to be a sensitive index of the excitability of the CNS and has the advantage that repeated measurements can be made over a relatively short period of time.

We are grateful to Dr D.T. Greenwood of I.C.I. Laboratories for helpful advice and discussion and to the Medical Research Council for financial assistance.

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Selective autoradiographic markers for GABA-releasing interneurones and nerve terminals

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In this light and electron microscopic autoradiographic study the cerebellum was used as a test model since only in this region of the brain has ultrastructure been fully correlated with function (Eccles, Ito & Szentagothai, 1967). In addition, neuropharmacological data is available to allow us to suggest that γ-aminobutyric acid (GABA) is the sole transmitter of the inhibitory interneurones of the cerebellum.

Either [^{3}H]-DABA (DL-2,4-diaminobutyric acid) or [^{3}H]-GABA dissolved in 2 μl of artificial CSF were microinjected under pressure into the centre of a single cerebellar folium of anaesthetized cats or rats at a depth of about 1 mm using a fine glass microelectrode (tip diameter <20 μm). Twenty minutes later the animals were killed by perfusion fixation with 5% glutaraldehyde. Subsequently 300 μm thick coronal slices of the lobule were cut on an Oxford 'vibratome' and prepared for electron microscopic autoradiography as described previously (Schon & Kelly, 1974).

Light and electron microscopic autoradiographs from the cerebellar folia following the microinjection of [^{3}H]-DABA (50 μCi) and [^{3}H]-GABA (25 μCi) provided similar results and were characterized by intensely and discretely labelled cell bodies in all layers of the cerebellum. At the electron microscopic level the labelled cells were identified as stellate cells in the molecular layer, as basket or Golgi cells in the Purkinje cell and upper granular layers. The Purkinje cells themselves, however, were virtually devoid of silver grains and their somas were silhouetted against a dense rim of radioactivity which became broader towards their rounded ends. Electron microscopic observations showed this dense rim to be composed of dense cluster of sliver grains located over unmyelinated axons and nerve terminals in synaptic contact with the Purkinje cell soma. These axons and terminals were tentatively identified to be basket cell axons or Purkinje cell recurrent collaterals.

Clusters of silver grains were also found in association with the cerebellar glomeruli; the silver grains were located exclusively over Golgi axon terminals in close contact with the granule cell dendrites. Neither the granule cell bodies or their dendrites nor the mossy fibre terminals were labelled.

In this study we have shown that light and electron microscopic autoradiographic techniques are now available to identify and map the distribution of the neurones in the mammalian CNS which use GABA as their transmitter. This technique is based on the hypothesis that such neurones possess a unique high affinity uptake site for the amino acids that they employ as their transmitter.

We are indebted to the Wellcome Trust for financial support to F.D.

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Topographical study of the distribution of GABA in the human substantia nigra

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In 1971, Precht & Yoshida first proposed the hypothesis that striatonigral fibres release GABA as a transmitter. Since then, interest has been focussed on the high concentrations of GABA which occur in the substantia nigra of most mammals and man (Perry, Hansen & Kloster, 1973). Since GABA, glutamic acid decarboxylase and the high affinity uptake of GABA appear to be specifically located in the nerve terminals of certain inhibitory neurons, it has been proposed that GABA in the substantia nigra may well be exclusively localized in nerve terminals. The main cellular components of the human substantia nigra are the melanin-rich cells which send their axons to the striatum and are believed to be dopaminergic in function. It would be of interest, therefore, to know the morphological distribution of GABA in the substantia nigra and its relationship to the dopaminergic neurons. In addition it now appears that a knowledge of the distribution of GABA in the substantia nigra could be of clinical value since a decreased level of GABA in the substantia nigra appears to be a feature of Huntingdon's chorea.

The midbrain from a neurologically normal 28-year-old male was obtained at autopsy within four hours of death. Using the method of Miyata & Otsuka (1972), transverse sections (150 μm thickness) of the rostral, middle and caudal substantia nigra were placed in a cold box (−20° C) and cut under a binocular microscope into 500 x 500 μm square blocks with a razor blade. The map of GABA distribution in each level of the substantia nigra was obtained by superimposing photographs taken before and after cutting the sections into square blocks.

In the rostral substantia nigra, the GABA distribution was markedly uneven, and the highest concentrations (more than 11.0 mM) were found in the pars reticulata. In the middle and caudal substantia nigra the GABA distribution was again uneven and the highest GABA levels were equally divided between the pars reticulata and the pars compacta. More detailed analysis of the results also supported the view that in the substantia nigra the highest concentrations of GABA are due to the presence of striato-nigral nerve terminals as they synapse with the dendrites of the nigral dopaminergic neurons whose cell bodies are located in the pars compacta (Rinvik & Grofova, 1970). In the cat more than half the synapses in the pars reticulata undergo degeneration following lesions of the ipsilateral caudate nucleus (Grofova & Rinvik, 1970) and the same kind of lesion in the rat is accompanied with a reduction in the GABA concentration of the substantia nigra.

I.K. was supported by grants from the Japan Society for Promotion of Science.

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Spontaneous and evoked release of newly synthesized [^{14}C]-GABA from rat brain slices

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After local perfusion of the monkey brain *in vivo* with [^{14}C]-glucose, newly formed [^{14}C]-amino acids, including GABA can be detected (De Feudis, Delgado & Roth, 1969). No method, however, for monitoring continuous release of newly synthesized amino acids *in vitro* has been reported The present technique was devised to measure the release of newly synthesized [^{14}C]-GABA from brain slices during perfusion with radioactive pyruvate as precursor.

Rat cortical slices, chopped in two directions at 0.3 mm intervals, weighing 100 mg, were placed in a Perspex tissue chamber and perfused at 37°C with Krebs bicarbonate medium containing [2-^{14}C]-pyruvic acid (specific activity 10 mCi/mmol), 2 μCi/ml, at a rate of 6 ml per hour. Perfusate samples were collected at 10 min intervals and stored on ice. At the end of the perfusion (90-150 min) the slices were homogenized in 0.1 N HCl and after centrifugation the supernatant fraction was used to estimate [^{14}C]-GABA remaining in the slices. In both perfusate and tissue samples [^{14}C]-GABA was estimated by the following procedure. Unchanged [2-^{14}C]-pyruvic acid and acidic products, including acidic amino acids, were removed by retention on a Dowex AG 1-X2 (CH_3COO^- form) anion exchange resin and the amino acid containing effluent collected Basic and neutral compounds in the effluent, including [^{14}C]-GABA, were retained on an Amberlite CG 120 (H^+ form) cation exchange resin, washed with water and eluted with 1 M triethylamine in 50% methanol. [^{14}C]-GABA in dried eluate samples was selectively isolated by enzymic conversion to [^{14}C]-succinic acid using a bacterial enzyme preparation as described by Hall, Bownds & Kravitz (1970). The reaction mixture was then passed again over an Amberlite CG 120 (H^+ form) cation exchange resin. Basic and neutral amino acids were unaffected by the enzyme reaction and were retained by the resin, [^{14}C]-succinic acid formed from [^{14}C]-GABA was collected in the effluent and radioactivity determined by liquid scintillation spectrometry.

The spontaneous release of newly synthesized [^{14}C]-GABA increased during the first 40 min of perfusion, after which a steady state was maintained. Contaminants derived from [2-^{14}C]-pyruvic acid in blank samples accounted for less than 10% of radioactivity in the [^{14}C]-succinic acid samples. Raising the potassium concentration in the perfusing medium to 47 mM produced a 229% ± 30% (mean ± s.e. mean, $n = 4$) increase in release The ratio of radioactivity remaining in the tissue at the end of perfusion, to the average basal efflux per 10 min collection period was 6.5 ± 0.9 (mean ± s.e. mean, $n = 7$). In contrast, when cortical slices were incubated with [^{3}H]-GABA for 30 min, followed by a washout perfusion, the amount of radioactivity remaining in the tissue after 60 min was over 100 times the average basal efflux per 10 min collection period.

Newly synthesized [^{14}C]-GABA may thus be released from a much smaller tissue pool than that labelled by [^{3}H]-GABA taken up from the external medium.

C.G. was supported by grants from the Royal Society and the CIBA Foundation. T.M.J. is an M.R.C. Scholar.

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A theoretical model of two functionally opposite receptor populations

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A theoretical model is presented to describe 'antagonistic agonism', i.e. the interaction between an agonist and two functionally opposite receptor populations.

The interaction between agonist A and the opposite receptors $R_{(+)}$ and $R_{(-)}$ can be broken down into three steps:

1. Relationship between agonist concentration ([A]) and biological stimuli ($S_{(+)}$ and $S_{(-)}$ (see Ariëns, Simonis & van Rossum, 1964a):

$$S_{(+)} = \frac{\alpha_{(+)}[A]}{K_{A(+)} + [A]}; \quad S_{(-)} = \frac{\alpha_{(-)}[A]}{K_{A(-)} + [A]} \qquad (1)$$

where $\alpha_{(+)}$ and $\alpha_{(-)}$ are the intrinsic activities of A on $R_{(+)}$ and $R_{(-)}$ respectively; $K_{A(+)}$ and $K_{A(-)}$ are the dissociation constants for the drug-receptor complexes $AR_{(+)}$ and $AR_{(-)}$ respectively.

Summation of stimuli:

$$S_t = S_{(+)} - S_{(-)} \qquad (2)$$

where S_t is the total biological stimulus.

3. Relationship between total biological stimulus and effect (E)

$$E = S_t - S_t \frac{S_{t\max} - E_{\max}}{S_{t\max}} \qquad (3)$$

where $S_{t\max}$ is the maximum total stimulus, and $E_{\max}$ is the maximum observable effect. (Equation (3) is formally similar to the equation proposed by Ariëns, Simonis & van Rossum (1964b) to describe functional synergism.)

Theoretical concentration-effect curves can be calculated by substituting into equations (1), (2) and (3). As the relationship between the two opposite biological stimuli is determined by the relative values of $\alpha_{(+)}$ and $\alpha_{(-)}$, and of $K_{A(+)}$ and $K_{A(-)}$, five different cases of antagonistic agonism can be distinguished. *Case 1:* $\alpha_{(+)} = \alpha_{(-)}$, $K_{A(+)} = K_{A(-)}$ $E = 0$ for all values of [A]. *Case 2:* $\alpha_{(+)} = \alpha_{(-)}$, $K_{A(+)} < K_{A(-)}$; the result is a bell-shaped concentration-effect curve. *Case 3:* $\alpha_{(+)} > \alpha_{(-)}$, $K_{A(+)} = K_{A(-)}$; the result is a monophasic saturation curve. *Case 4:* $\alpha_{(+)} > \alpha_{(-)}$, $K_{A(+)} < K_{A(-)}$; the result is a monophasic curve which has a peak, and then declines to a plateau. *Case 5:* $\alpha_{(+)} > \alpha_{(-)}$, $K_{A(+)} > K_{A(-)}$; the result is a biphasic curve, consisting of a negative and a positive phase The receptor population whose activation is reflected in the direction of the observed effect, may be called 'dominant', and the opposite receptor population may be called 'masked'.

A competitive antagonist acting at either or both receptors can change the relationship between the two component concentration-stimulus curves. Thus the blockade of masked receptors causes potentiation, and the blockade of dominant receptors causes reversal of the response If both receptors are affected by the antagonist, either potentiation or antagonism can be observed depending on the concentrations of the agonist and antagonist.

This model is used to explain the unmasking of latent effects (Tamayo, Contreras & Quijada, 1974), bell-shaped dose-response curves (Okpako, 1972), biphasic dose-response curves (Spedding & Weetman, 1972), potentiation by antagonists (Ignarro & Titus, 1968), reversal by antagonists (Ignarro & Titus, 1968), and the dual action of antagonists (Burks & Cooper, 1967).

I am grateful to C.M. Bradshaw and P Bevan for helpful discussions.

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A simple and cheap method of screening glass microelectrodes

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The recording of fast intracellular potential changes during micro-iontophoretic studies is complicated by the attenuation of high frequency responses by the distributed capacitance between the recording electrode and the multibarrelled iontophoretic electrode assembly. This attenuation is particularly troublesome in coaxial electrodes of the type described by Sonnhof (1973). The problem can be overcome by the use of screened recording electrodes—the screen being driven from the input amplifier at unitary gain. Provided that the input amplifier has good HF characteristics, this method will also allow accurate measurement of cell membrane resistance by current pulses through the recording electrode (cf. Eide, 1968). An efficient method to screen electrodes was developed by Eide, Engberg & Sonnhof (see Sonnhof, 1973), but this necessitates access to expensive high vacuum equipment for 'sputtering' a thin layer of gold onto the micropipettes. Further the electrodes must be filled with electrolyte *after* this goldplating when their interior is no longer visible. To avoid these complications we have used the following procedure.

Single, *filled*, electrodes were coated to within 1 mm of the tip with graphite from an aerosol can (Graphit 33, Kontakt Chemie, Rastatt, W. Germany). The electrode was held in a clamp so that the tip dipped into 99% ethanol and then sprayed so that an even layer of graphite was deposited on the glass. The coated electrode was then inserted into the central canal of the iontophoretic assembly, where it was surrounded with a small amount of saline. The graphite screen *or* the saline was connected to a unitary gain output of the amplifier output via a 100 nF capacitor.

The present technique has proved to be quite comparable to the gold screening in conjunction with coaxial multibarrel electrodes (for illustration see Figure 1 of the communication by Engberg, Flatman & Lambert, 1975, this meeting). Graphite screening can also be used to neutralize the capacitance of single electrodes in other types of recording, if the screen is insulated by a thin layer of lacquer covering the part that enters the tissue or conducting fluid.

We acknowledge the technical assistance of Mr A Jensen and a travelling grant from the Wellcome Trust to J.D.C.L.

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Combined EEG recording and intraventricular administration of drugs in the conscious rat

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This technique, a modification of that described previously by Hill & Miller (1973) for use in the rabbit, allows the combination of EEG recording and intraventricular administration of drugs in the conscious rat.

EEG activity is recorded from a 4-pin miniature electrical connector (ITT Cannon, Basingstoke) linked to stainless steel screws inserted in the skull. The small dimensions of the connector (1.5 mm x 6 mm x 9 mm high) and especially the narrow width are of considerable advantage for use on the rat skull.

The intraventricular cannula was modified from that described by Hayden, Johnson & Maickel (1966). It was prepared from 3 mm diameter Perspex rod, the small diameter being of advantage for combination with the electrical connector.

The technique may also be combined with behavioural activity recording using ultrasonic transducers as described by Hill & Miller (1973).

The implantation of the connector, cannula and screws was performed under halothane anaesthesia. Rats prepared by this method have shown no abnormal behaviour and have provided satisfactory EEG recordings over several months.

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A centrally-acting antihypertensive agent R28935, a pimozide analogue, not acting via α-adrenergic neurones

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Clonidine and α-methyldopa (Schmitt, Schmitt & Fenard, 1973; Finch, 1974; Finch & Haeusler, 1973a; Van Zwieten, 1975) are known to exert their antihypertensive action via stimulation of central α-adrenoceptors. However, the role of dopaminergic neurones in the central regulation of blood pressure remains unclear, since the potent dopamine agonist apomorphine lowers blood pressure with an accompanying bradycardia in both the anaesthetized cat and rat (Barnett & Fiore, 1971; Finch & Haeusler, 1973b), whilst dopamine administered intraventricularly in the conscious cat has been reported to exert a hypertensive action (Day & Roach, 1974).

Erythro-1-{1-[2-(1,4-benzodioxan-2-yl)-2-OH-ET]-4-piperidyl}-2-benzimidazolinone (R.28935), structurally related to the neuroleptic pimozide (Wellens, Van Neuten & Janssen, 1975) when administered intraventricularly (i.c.v.) lowered blood pressure in conscious renal hypertensive cats for a period of 5 h without altering the resting heart rate. Pretreatment with α-adrenoceptor blocking agents phentolamine (200 μg, i.c.v.) or tolazoline (200 μg, i.c.v.) failed to modify the hypotensive action of R.28935 (25 μg, i.c.v.). Similar pretreatments with tolazoline and phentolamine completely abolished the hypotensive effects of α-methyldopa (1 mg, i.c.v.) or clonidine (20 μg, i.c.v.) respectively.

Peripheral administration of haloperidol (1-5 mg/kg i.p.) and pimozide (1-2 mg/kg i.p. or 100-200 μg, i.c.v.) produced a dose-dependent fall in blood pressure and associated bradycardia which was also accompanied by stereotyped behaviour. However, administration of R.28935 (0.05-0.1 mg/kg i.p.) produced a dose-dependent fall in blood pressure without any accompanying bradycardia and no behavioural changes were detected.

In the Krebs-perfused mesenteric artery preparation, R.28935, in doses of 0.1-5 mg, did not exert any vasoconstrictor action whilst clonidine (0.1-1 mg) exhibited a dose-dependent vasoconstrictor effect. Infusions of R.28935 (0.1-10 mg) exhibited a dose-dependent vasoconstrictor actions of noradrenaline, 5-hydroxytryptamine or ATP. The vasoconstrictor action of clonidine was completely abolished by infusions of phentolamine (0.5 mg/l).

From the results it is concluded that R.28935 is a novel centrally-acting antihypertensive agent not acting via central α-adrenoceptors and is devoid of peripheral sympathomimetic properties. Similar observations have been reported in studies using the anaesthetized cat, dog and rabbit ear artery preparations (Van Zwieten, 1975; Wellens, Van Neuten & Janssen, 1975).

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Evaluation of narcotic and narcotic antagonist analgesic drugs in the dog dental pulp stimulation test

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In antinociceptive tests based on electrical stimulation of the dental pulp it is difficult to identify the response to electrical stimulation and to quantify the degree to which this response is changed by drugs. In the present work a procedure in the conscious dog is described in which the effects of drugs and placebo were measured in terms of changes in the stimulus threshold for eliciting a minimal response to dental pulp stimulation. Using this procedure reproduceable, dose-dependent increases in stimulation threshold can be obtained with narcotic and narcotic antagonist analgesic drugs.

Adult, male beagle dogs were trained to sit in well ventilated individual boxes (32 x 23 x 28 in high). The boxes were illuminated inside and were fitted with a clear plate glass door for observation of the dogs. Following a training period, which ranged from 1-4 weeks, stainless steel stimulation electrodes were implanted in an upper canine tooth under pentobarbitone anaesthesia, using an operative technique similar to that described by Neat & Peacock (1971). Leads from the electrodes were passed subcutaneously to an external connector located on the back of the neck. The dogs were ready for testing 7-10 days post-operatively.

All tests were carried out such that the operator did not know whether a dog had received a drug or placebo. Each dog received one placebo and one drug trial each week. Drugs were administered either orally in gelatin capsules or subcutaneously. Groups of at least five dogs were used for each dose-level. Electrical stimulation thresholds (0.5-15 V) to cause a characteristic, minimal response (i.e. licking, chewing, head movement, etc.) were determined for each dog prior to, and then at 30 min intervals up to 3 h after drug administration. Stimulus trains were delivered from a Devices isolated stimulator using square wave pulses of 5 ms pulse width, 10 Hz frequency and 10 s train duration, at least 30 s was allowed between trains of impulses during threshold determinations. The resistances of the tooth pulp electrodes (4-30 k) were monitored continuously on an oscilloscope as a check for open circuits.

The mean change (± s.e.) in stimulation threshold during placebo trials was $-0.14 \pm 0.41\%$ ($n = 20$). Maximum drug-induced changes in stimulation thresholds ranged from +35 to +40%. Statistically significant ($P = 0.05$), dose-dependent increases in stimulation thresholds were obtained with subcutaneous doses (mg/kg) of pentazocine (0.25-2) and nalorphine (0.1-1) and with oral doses (mg/kg) or morphine (0.25-3) and codeine (2-5). The potencies of these analgesic drugs were therefore similar to those found clinically. Results obtained with aspirin (100 mg/kg orally) were inconsistent. The following non-analgesic drugs (mg/kg orally) were inactive: atropine (1.0), mepyramine (0.5), phentolamine (1.0) and propranolol (1.0).

In our experience, dental pulp stimulation in the beagle dog provides an accurate and reproduceable model for the evaluation of the antinociceptive activities of narcotic and narcotic antagonist analgesic drugs.

We are grateful to Mr R. Peacock for carrying out the electrode implantation operations and to Mr D. Harrison for constructing the electrode connectors.

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Simple models of anaphylaxis and of histamine and 5-hydroxytryptamine induced inflammation using the mouse pinna

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The mouse pinna may be used as a site for the study of allergic and inflammatory reactions. We demonstrate its use in two roles: firstly, pinnal anaphylaxis as a model of an acute allergic response; second, the inflammatory action of histamine and 5-hydroxytryptamine on the mouse pinna. Both systems provide simple, rapid and cheap methods for either drug screening or university class demonstrations.

The sensitization procedure for pinnal anaphylaxis has been described previously (Church, James & Miller, 1974). Male CFLP mice were injected subcutaneously with 0.1 ml of a 1/20 dilution followed fourteen days later by a further 0 1 ml of a 1/50 dilution of horse serum. The animals were challenged six to eight days after the second dose of horse serum.

Challenge was performed in a warm room (30-32°C) to which the animals were taken one hour previously to acclimatise. This environment facilitates rapid intravenous injections but does not significantly change the development of the reaction. Mice were lightly anaesthetized with ether and injected intravenously with 0.2 ml of 1% Evans blue solution. Immediately after this and while still anaesthetized, the mice were laid on their back and each pinna stabbed with a 21 gauge hypodermic needle through a drop of antigen (neat horse serum) or agonist dissolved in 0.9% sodium chloride solution. Thirty minutes were allowed for the subsequent blueing reaction to develop before the mice were killed and their ears cut off and mounted on cards. The reaction was measured as the area of blueing around the site of challenge.

Challenge of sensitized mice with antigen produced a sub-maximal reaction which is subject to diurnal variation, peak sensitivity occurring at 16.00 h and minimum sensitivity at 10.00 h (Miller & Church, 1975).

In non-sensitized mice, 5-hydroxytryptamine was more potent than histamine in inducing inflammation. The concentrations of agonists used to induce approximately equal sub-maximal responses were 0.2 mg/ml of 5-hydroxytryptamine and 1 mg/ml of histamine.

Pinnal anaphylaxis was partially inhibited by anti-histamine and anti-5-hydroxytryptamine agents and was reduced by anti-inflammatory drugs, β-adrenoceptor stimulants, phosphodiesterase inhibitors and corticosteroids but not disodium cromoglycate. Histamine induced inflammation was inhibited by histamine H_1-receptor antagonists injected subcutaneously 20 min before test, e.g. mepyramine (ID_{50}: 1.34 mg/kg) and diphenhydramine (ID_{50}: 2.51 mg/kg). Histamine H_2-receptor antagonists and 5-hydroxytryptamine antagonists were not active. 5-Hydroxytryptamine-induced inflammation was inhibited by specific antagonists administered similarly, e.g. methysergide (ED_{50}: 0.008 mg/kg) but not by antihistamines. Cyproheptadine administered similarly inhibited both histamine and 5-hydroxytryptamine induced inflammation (ID_{50}: 0.024 mg/kg and 0.16 mg/kg respectively).

We wish to thank May & Baker, Wellcome Research Laboratories, Smith, Kline & French, ICI and Merck, Sharp & Dohme for the gifts of compounds used in these studies.

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Analgesics and rotational behaviour in rats with unilateral substantia nigra lesions. Effects in the presence and absence of (+)-amphetamine

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It has previously been reported that morphine and the narcotic antagonist analgesics, buprenorphine and pentazocine, antagonize apomorphine-induced turning behaviour in rats with lesions (caused by 6-hydroxydopamine) in the left substantia nigra (Cowan, Dettmar & Walter, 1975). The three analgesics have now been tested in the same behavioural model against (ipsilateral) turning evoked by (+)-amphetamine (Ungerstedt, 1971). At least 28 days after surgery, the colony of male Sprague-Dawley rats (initially 180-200 g) was injected with (+)-amphetamine sulphate (2 mg/kg, i.p.). Fifteen minutes later, each rat was individually placed in a 'rotometer' and the incidence of circling was monitored for 50 minutes. The 'rotometer', which displays the number of clockwise and anticlockwise turns of 6 rats over any pre-set time interval of up to 99 min, will be demonstrated.

After at least 7 days, groups of 8-10 rats received s.c. either physiological saline solution or one of a series of doses of buprenorphine, morphine or pentazocine. Fifteen minutes later, the animals were challenged with (+)-amphetamine or saline and, a further 15 min later, the extent of circling was recorded for 50 minutes.

In the absence of (+)-amphetamine, buprenorphine (0.01-0.30 mg/kg), morphine (0.30-3 mg/kg) and pentazocine (1-10 mg/kg) increased ipsilateral turning in a dose-related manner. Smaller increases were obtained with higher doses of buprenorphine (1 mg/kg), morphine (10 mg/kg) and pentazocine (30 mg/kg). Significant differences in turning were obtained, in relation to saline-injected controls, with rats receiving each of the doses of buprenorphine, morphine at 1 and 3 mg/kg and pentazocine at 3, 10 and 30 mg/kg ($P < 0.05$, Mann Whitney U test).

Buprenorphine caused a significant reduction in D-amphetamine-induced turning at 0.10 and 0.30 mg/kg ($P < 0.05$) but not at 0.03 and 1 mg/kg. With morphine (0.03-10 mg/kg) and pentazocine (0.10-30 mg/kg) a significant reduction in turning only occurred with the top dose of each compound ($P < 0.05$).

The ability of the same doses of buprenorphine to both cause turning and antagonize D-amphetamine-induced turning suggests an unusual involvement of this new analgesic with the nigrostriatal system.

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Thrombolytic and anti-thrombotic properties of dihomo-γ-linolenate *in vitro*

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Arterial thrombosis is thought to be precipitated by intravascular platelet clumping (Mustard & Packham, 1975) and vasoconstriction could be a complicating factor.

Prostaglandin E_1 (PGE_1 has very desirable actions as a potential anti-thrombotic drug, since it can completely prevent aggregation of platelets induced by large concentrations of any aggregating agent (Kloeze, 1967; Chandra Sekhar, 1970). It thus has advantages over aspirin which can only inhibit that component ('second phase') of the aggregation response that is mediated through enzymatic conversion of platelet arachidonate to prostaglandin endoperoxide (Willis, 1974a, b; Hamberg, Svensson, Wakabayashi & Samuelsson, 1974; Willis, Vane, Kuhn, Scott and Petrin, 1974).

Prostaglandin E_1 has not been used as an anti-thrombotic agent because of its rapid inactivation (Ferreira & Vane, 1967) and expected variety of severe side effects. However, it has recently been proposed that administration of the PGE_1 precursor, dihomo-γ-linolenate (DHLA) might overcome these drawbacks, since PGE_1 would then be produced as required at its site of action, without high circulating levels of PG (Willis, Comai, Kuhn & Paulsrud, 1974; Willis, 1975). It is often overlooked that PGE_1 can also produce *lysis* of platelet aggregates (Chandra Sekhar, 1970). We thus examined whether DHLA has similar 'thrombolytic' properties.

Citrated platelet-rich plasma (PRP) from man, or heparinized PRP from rat was prepared by centrifugation (Weiss, Aledort & Kochwa, 1968) and aggregation studied at 37°C in a dual channel aggregation module (Payton Associates). Arachidonic acid (AA) and DHLA ($>$ 99% pure, Nucheck) were dissolved at 10 mg/ml in dimethylsulphoxide (DMSO, BDH), and kept under nitrogen. Prostaglandins E_1 and E_2 (Upjohn) were dissolved at 20 μg/ml in 0.9% sodium chloride solution. Routinely, arachidonic acid was used as the aggregation stimulus since it has a role in irreversible platelet aggregation. However, basic features of the study were confirmed using large concentrations ($>$ 1 μg/ml) of ADP. The findings described were repeated on four or more separate occasions.

Simultaneous administration to PRP of equimolar concentrations of AA and DHLA (100-300 μg/ml) always resulted in a competitive effect, so that the maximal aggregation response to AA was always reduced (20-90%) but the most marked effect was a virtually complete reversal of the AA-induced aggregation in the presence of DHLA. DHLA also produced partial (40-80%) reversal of aggregation if added when the aggregation response had largely or completely developed. Visual and microscopic examination of the PRP showed that the aggregates had been considerably reduced in size and number.

The ability of DHLA to reverse the aggregation response is probably attributable to its bioconversion to PGE_1 within the platelets, since PGE_1 (1-3 μg/ml) but not PGE_2 could also reverse aggregation induced by AA or ADP. Also ability of DHLA to produce this reversal of aggregation was blocked in the presence of aspirin (40 μg/ml), although that of PGE_1 was not. Aspirin could not itself alter aggregation which had already developed.

Intravenous injection of DHLA (2 mg/kg) in dogs decreases platelet aggregation (Rose, Johnson, Ramwell & Kot, 1975). Our results suggest that PGE_1 may be generated locally in the area of incipient-infarction, where it may lyse platelet thrombi which had already formed, prevent and reverse further aggregation of platelets, and produce vasodilatation which could make infarction less likely.

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Printed in Great Britain by The Whitefriars Press Ltd, London and Tonbridge

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British Journal of Pharmacology

November 1975

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The British Journal of Pharmacology is published monthly by Macmillan Journals Ltd, 4 Little Essex Street, London WC2R 3LF. Telephone: 01-836 6633. ISSN 0007-1188.

Annual subscription £36.00 ($90 USA and Canada). (Orders must be accompanied by remittance. Prices applicable only to orders received before 31st December 1975.)

Cheques should be made payable to Macmillan Journals Limited: Subscription Department, Brunel Road, Basingstoke, Hampshire RG21 2XS, England.

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Second class postage paid at New York, N.Y.
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EFFECT OF AMODIAQUINE ON GASTRIC HISTAMINE METHYLTRANSFERASE AND ON HISTAMINE-STIMULATED GASTRIC SECRETION

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1 Amodiaquine was found to be a potent inhibitor *in vitro* of gastric histamine methyltransferase from human and canine corpus and from pig antrum. The ID_{50} for the enzyme, purified from pig antrum mucosa by ultracentrifugation and chromatography on DEAE-cellulose, was 2.5 μM.

2 In six dogs with Heidenhain pouches the maximum secretory response to histamine (40 μg/kg i.m.) was augmented by i.m. injection of amodiaquine. The augmentation depended on the dose of amodiaquine, the optimum effect (40% increase in volume of gastric juice, 80% in acid output) being achieved with 2 mg/kg. The maximum secretory response to betazole was also enhanced by amodiaquine.

3 It was suggested that amodiaquine may enhance the histamine and betazole stimulated gastric secretion by an inhibition of gastric histamine methyltransferase *in vivo*.

Introduction

The stimulation of gastric acid secretion by pharmacological doses of histamine, the occurrence of histamine both in gastric juice and tissue and the release of histamine by gastrin in the rat gastric mucosa predominantly support the hypothesis that histamine is a physiological stimulator of gastric secretion (Code, 1956; Kahlson, Rosengren, Svahn & Thunberg, 1964; Lorenz & Pfleger, 1968). Although this hypothesis has been rejected by several authors (Grossman, 1967; Johnson, 1971), others have continued to defend the idea that histamine may be the common mediator for the action of gastrin and acetylcholine on the parietal cell (Code, Maśliński, Mossini & Navert, 1971; Kahlson & Rosengren, 1971). These differences in opinion were based on disagreements about subordinate elements in the hypothesis: adequate localization of histamine in the mucosa (*pro*: Thunberg, 1967; *contra*: Håkanson, 1970), release of histamine during physiological stimulation of acid secretion (*pro*: Kahlson *et al.*, 1964; *contra*: Håkanson, 1970) and formation of histamine in the gastric mucosa of various mammalian species (*pro*: Kahlson *et al.*, 1964; Lorenz, Halbach, Gerant & Werle, 1969; *contra*: Aures, Davidson & Håkanson, 1969).

However, there is good agreement about the inactivation of histamine in the stomach. High activities of histamine methyltransferase (S-adenosyl-methionine: histamine-*N*-methyltransferase; EC 2.1.1.8) were demonstrated in man and all animals investigated (Brown, Tomchick & Axelrod, 1959; Lorenz, Barth & Werle, 1970a), but no, or only a very low, activity of diamine oxidase (diamine: oxygen-oxidoreductase EC 1.4.3.6) could be measured in the gastric mucosa of various mammals, including rats, dogs and cows (Kusche, Lorenz, Hahn & Werle, 1969; for a review see Lorenz *et al.*, 1970a).

Therefore we attempted to find a potent inhibitor of gastric histamine methyltransferase *in vitro* and then to investigate its effect on histamine-stimulated gastric secretion; the antimalarial drug amodiaquine was already known to be an inhibitor of this enzyme from the rat kidney (Cohn, 1965). A preliminary report of our findings has been published (Troidl, Lorenz, Barth, Seidel, Rohde, Goecke, Schmal & Hamelmann, 1972).

Methods

Source and preparation of histamine methyltransferase

Gastric mucosa from duodenal ulcer patients (treated by a Billroth II gastrectomy), from dogs (laboratory animals) or from pigs (slaughter house) was rinsed with ice-cold Tyrode solution immediately after removal of the stomach. The mucosa was stripped off from the muscular layer, frozen with dry ice and then stored at –20° C for several days.

Crude extracts of histamine methyltransferase were prepared by homogenizing the frozen tissues with 2 vol 0.25 M sucrose solution. The homogenate was centrifuged at 2° C and 100,000x *g* for 30 min and the supernatant was used as the source of the enzyme. Histamine methyltransferase from pig antrum mucosa was partially purified by applying the crude extract to a column of DEAE-cellulose (Lorenz, Reimann, Barth, Kusche, Meyer, Doenicke & Hutzel, 1972).

The fractions containing the highest enzyme activities were combined and used directly for incubation. The purification of this enzyme preparation was 22-fold as compared with the crude homogenate, the specific activity being 4.04 nmol/(min x mg protein) with a yield of 74%. The proteins were determined according to Lowry, Rosebrough, Farr & Randall (1951).

Reagents

Histamine dihydrochloride puriss. (Fluka, Buchs); 2,5-diphenyloxazole (PPO), 2,2′-p-phenylene-bis-(5-phenyloxazole) (POPOP) and toluene (all scintillation grade), isoamylalcohol p.a., D(+)-sucrose puriss., nicotinamide p.a., Folin-Ciocalteu's phenol reagent, 0.1 N sodium hydroxide solution (Titrisol), all inorganic salts, acids and bases p.a. (Merck, Darmstadt); S-adenosyl-L-(^{14}C-methyl)-methionine (0.51 mCi/mmol) (The Radiochemical Centre, Amersham); S-adenosyl-L-methionine hydrogen sulphate (Boehringer, Mannheim); amodiaquine (kindly supplied by R. Wolf, Parke & Davies, Munich); DEAE-cellulose-SS p.a. (Serva, Heidelberg). All solutions were prepared with twice-distilled water.

Drugs

Sterile solutions of histamine dihydrochloride and amodiaquine were prepared by the University's pharmacy; betazole dihydrochloride (Histalog, Lilly, Giessen); insulin (Hoechst, Frankfurt).

Cannula for the Heidenhain pouch experiments

The experiments were planned to last many months and so, to minimize the risk of technical failure, a special tissue-sparing corrosion-resistant cannula was developed based on the approved models of Dragstedt, Haymond & Ellis (1933), Gregory (1950) and DeVito & Harkins (1959). This cannula consisted of a stainless steel tube placed on a shaft of polypropylene. A relatively broad plexiglass disk, pushed over the stainless steel tube, was fixed on one side by the polypropylene shaft and on the other side by the interior surface of the abdominal wall (Emås, Swan & Jacobson, 1967). The disk was intended to shunt off the strong pulling and shearing forces which act occasionally on the exterior part of the cannula in the lying animal and so prevent the cannula from breaking out of the pouch.

Determination of histamine methyltransferase activity in vitro

The enzyme activity was determined by the isotope assay of Barth, Lorenz & Niemeyer (1973a) using S-adenosyl-L-[^{14}C-methyl]-methionine as the methyl-group donor. Amodiaquine, dissolved in 0.1 ml of 0.05M sodium phosphate buffer at pH 7.4, was added to the incubation mixture, which contained 32-48 nmol histamine, 250 nmol S-adenosyl-L-methionine, 100 nmol S-adenosyl-L-[methyl ^{14}C]-methionine and 22.5 μmol phosphate buffer, pH 7.4. The final volume of the incubation mixture was 0.5 ml. The incubation was stopped after 0, 15, 30, 45 and 60 min by the addition of borate buffer, pH 10.0.

The reaction product, [^{14}C]-1-(τ)-methylhistamine, was extracted into a mixture of toluene and isoamylalcohol. After the addition of scintillant, an aliquot was counted in a Tri Carb liquid scintillation spectrometer. The formation of 1 nmol of 1-methylhistamine corresponded to 114 ct/minute. The observed specific activities of histamine methyltransferase lay between 98 and 574 pmol/(min x mg protein), corresponding to counts of values of 1470-4350 ct/min over 60 minutes. Depending on incubation time, the blanks with heat-inactivated enzyme showed values of 80-150 ct/min over 60 min above the background of 30 ct/minute.

Evaluation of the effect of amodiaquine on histamine-stimulated gastric secretion in the Heidenhain pouch of dogs

The effect of amodiaquine on histamine and betazole stimulated gastric acid secretion was

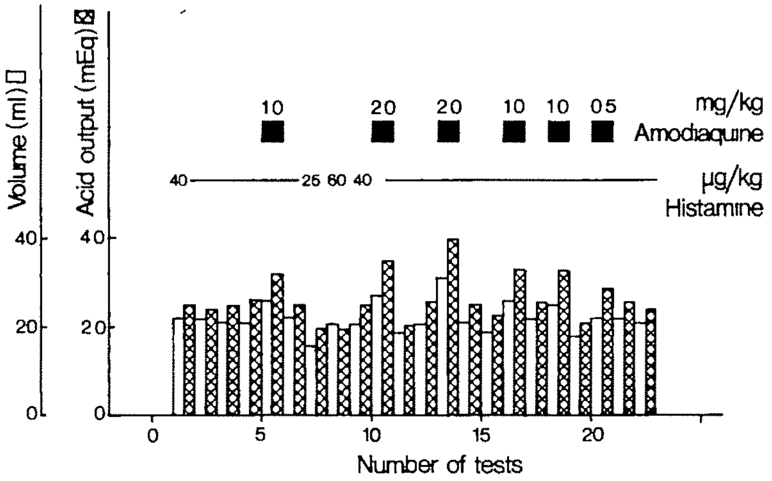

Figure 1 Enhancement of the histamine-stimulated gastric secretion in a Heidenhain pouch dog by amodiaquine. From the test programme 11 weeks are shown, including the intial tests for reproducibility, several doses of histamine and of amodiaquine according to the test procedure (see test). Histamine is expressed as histamine dihydrochloride. The results are those for total gastric secretion (volume and acid output/secretion period). Dog No. 2, male, 23.5 kg at the time of operation (12.7.1971). ☐ volume, ▨ acid output. For futher conditions see methods section.

investigated in six mongrel dogs (20-30 kg, both sexes) with Heidenhain pouches. The preparation of these pouches, the supervision of the dogs after the operation and during the test period, and the experimental design for the stimulation of gastric secretion by secretagogue alone or by secretagogue and amodiquine (cf. Figure 1) in serial tests were performed as described by Troidl, Lorenz, Barth, Rohde, Feifel, Schmal, Goecke, Reimann-Huhnd & Seidel (1973).

Additionally every six months, insulin (0.2 i.u./kg) was used to check the lack of vagal reinnervation of the pouches. During the first 90 min of this test the blood sugar concentration was determined every 15 min by the glucose oxidase assay (Boehringer-Kit, Mannheim). The result of the insulin test was only considered to be satisfactory if the glucose concentration fell to values between 30 and 10 mg/100 ml blood (Baron, 1970).

The procedure for a single test was the same as described by Troidl *et al.* (1973), using 40 µg/kg histamine or 2 mg/kg betazole instead of pentagastrin. Gastric juice was collected in 30 min-portions for 2.5 to 3 h, after which time in all experiments the basal secretion plateau was attained. Determination of the volume and the acidity of each sample was carried out as described by Troidl *et al.* (1973).

Definitions

The maximum secretory response to histamine and betazole was defined by two parameters, the total secretion and the peak secretion. The total secretion consisted of all fractions of gastric juice obtained within the 2.5 to 3 h of secretion and was expressed as ml and mEq/secretion period. The peak secretion was represented by those two consecutive 30 min fractions, irrespective of time, which showed the greatest volumes and acid outputs and was expressed in ml and mEq/hour. The effect of amodiaquine was expressed as the ratio of the results of the single test with amodiaquine plus stimulant to the average results of the two control tests without amodiaquine (Troidl *et al.*, 1973). Augmentation or inhibition of the stimulated secretion by amodiaquine was expressed as a percentage, because the variation of the secretion values was relatively high from animal to animal.

Statistical calculations

Statistical calculations (Snedecor & Cochran, 1967) including those of mean values, coefficient of variation, standard deviations and tests for statistical significance (Student's t-test or t-test for paired data) were performed with the aid of a desk computer (Olivetti Programma 102).

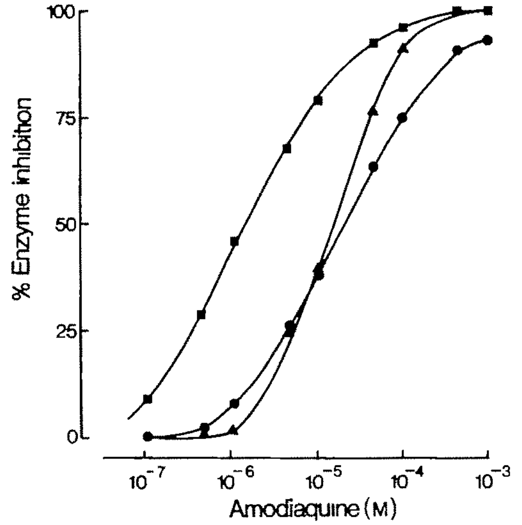

Figure 2 Inhibition of gastric histamine methyltransferase by amodiaquine. The enzyme activity in the absence of the inhibitor, which equalled 100%, was 0.3 nmol methylhistamine formation/min and mg protein for the pig (■), 0.25 nmol/min and mg for the dog (●) and 0.1 nmol/min and mg for the human gastric mucosa (▲). Mean values from three determinations. For further conditions see methods section.

Results

Inhibition of gastric histamine methyltransferase by amodiaquine in vitro

Amodiaquine was found to be a very potent inhibitor of histamine methyltransferase from human and canine corpus mucosa and from pig antrum mucosa (Figure 2). The ID_{50} value using pig enzyme was smaller by one order of magnitude than found with the enzymes from man or dog. Crude and partially purified enzyme preparations were inhibited by amodiaquine to the same extent, since the ID_{50} for histamine methyltransferase following chromatography on DEAE-cellulose was 2.5 μM (cf. Figure 2). A 30 min preincubation of amodiaquine and the enzyme at 37° C enhanced the inhibitory effect by 20% at inhibitor concentrations smaller than 50 μM.

Effect of amodiaquine on the histamine and betazole stimulated gastric secretion in Heidenhain pouch dogs

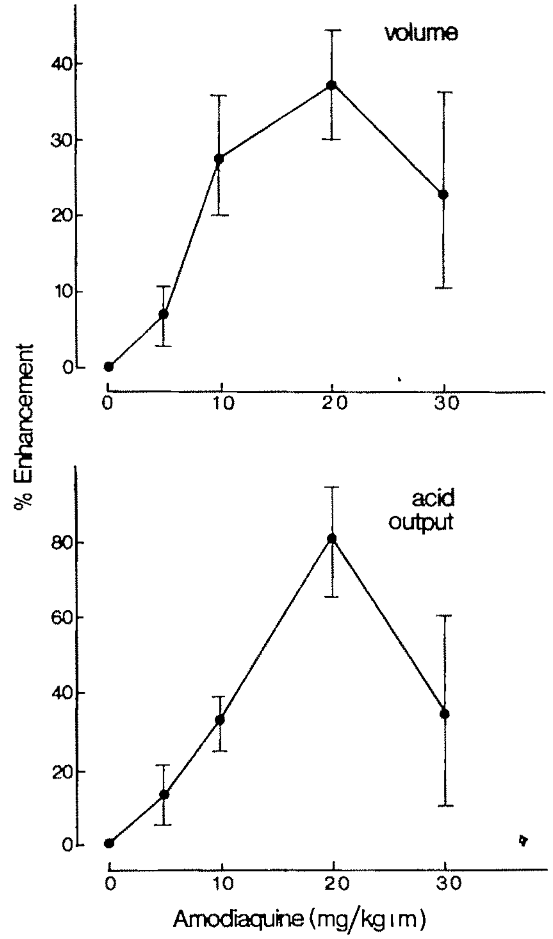

Figure 3 Enhancement of the histamine-stimulated gastric secretion in Heidenhain pouch dogs by amodiaquine. Mean values ± s.e. mean from 6-11 tests per dose of amodiaquine. Dose of histamine dihydrochloride 40 μg/kg i.m., values given as total gastric secretion. Zero means the histamine-stimulated secretion without injection of amodiaquine (34 ± 17 ml and 3.8 ± 1.9 mEq/secretion period $\bar{x}$ ± s.d.). For further conditions see methods section.

In six dogs, the maximum secretory response to histamine (40 μg/kg i.m.) showed an excellent reproducibility (for an example see Figure 1). The total volume was 34 ± 17 ml, the total output 3.8 ± 1.9 mEq/secretion period (x ± s.d.). Amodiaquine enhanced the total gastric secretion (Figure 3). The optimum dose was 2 mg/kg which enhanced the volume of gastric juice by 40% and the acid output by 80%. A higher dose of amodiaquine (3 mg/kg) produced a less marked increase and in three dogs, 5 mg/kg inhibited the total gastric secretion by about 50%. Weakness and apathy were observed in these dogs indicating that this dose was toxic (Lorenz, Thermann, Messmer, Schmal, Dormann, Kusche, Barth, Tauber, Hutzel, Mann & Uhlig, 1974). Amodiaquine augmented the peak secretion (24 ± 9 ml/h, 2.8 ± 1.0 mEq/h) and the total secretion to the same degree. In all experiments, amodiaquine (2 mg/kg) enhanced the peak volume of gastric juice by 37 ± 18%, the peak acid output by 82 ± 31%. The total volume of the gastric juice increased by 36 ± 16%, the total acid output by 80 ± 33%. This indicates that histamine-stimulated gastric secretion was enhanced but not prolonged by amodiaquine. The secretion period following stimulation was 120-150 min both in experiments with histamine alone and in those using the combined stimulation by histamine plus amodiaquine.

The histamine isomer betazole, which has been used as a stimulator of human gastric sectretion (Feifel, Lorenz, Heimann & Wörsching, 1972) was administered in two dogs in combination with amodiaquine. In four experiments, the maximum gastric secretion evoked by 2 mg/kg i.m. betazole (total volume 68 ml, total acid output 7.5 mEq/secretion period), was enhanced by 24 ± 10% (volume) and 45 ± 18% (acid output) after amodiaquine (2 mg/kg). The augmentation of the peak secretion by amodiaquine did not differ significantly from that of the total secretion.

Discussion

Antimalarial drugs derived from 4-aminoquinoline were reported to be potent *in vitro* inhibitors of catechol-*O*-methyltransferase (Spector, personal communication; see Cohn, 1965) and histamine methyltransferase from rat kidney (Cohn, 1965). The ID_{50} values were smaller by 1-3 orders of magnitude than those of other inhibitors of the latter enzyme, such as 1-(τ)-methylhistamine (Brown *et al.*, 1959; Lindahl, 1960; Barth *et al.*, 1973a), 5-hydroxytryptamine (Brown *et al.*, 1959; Gustafsson & Forshell, 1964), chlorpromazine and bromolysergic acid diethylamide (Brown *et al.*, 1959), Cu^{2+}-ions (Lindahl, 1960; Gustafsson & Forshell, 1964), antihistamines of the H_1-receptor type (Netter & Bodenschatz, 1967; Cohn & Wynn, 1968; Barth, Niemeyer & Lorenz, 1973b, c) and H_2-receptor antagonists (Barth *et al.*, 1973b, c). We have now shown that amodiaquine is a potent inhibitor of histamine methyltransferase of the gastric tissue of man, dog and pig.

In vivo, investigations on the inhibition of histamine methyltransferase by amodiaquine were performed in rats by studying the daily excretion of histamine in the urine (Cohn, 1966; Kim & Cohn, 1966). The increase of the histamine excretion which was observed has to be interpreted primarily as an inhibition of the renal histamine methyltransferase, since high activities of this enzyme were found in rat kidneys. However, these findings emphasized that amodiaquine was capable of inhibiting the histamine methyltransferase *in vivo*. Therefore, the enhancement by amodiaquine of gastric secretion in dogs stimulated by histamine, or betazole which releases histamine (Stubrin, Dyce, Brem, Tecimer & Haverback, 1965; Lorenz, Feifel, Schmal, Hutzel & Werle, 1970b) may be explained as an inhibition of the high activities of this enzyme in the fundic and corpus mucosa (Lorenz *et al.*, 1970a).

It has previously been shown that pentagastrin-stimulated secretion is also augmented by amodiaquine (Troidl *et al.*, 1972, 1973). Studies on the *in vivo* inhibition of gastric histamine methyltransferase by agents other than amodiaquine were performed in rats by Amure & Ginsburg (1964a, b), using chlorpromazine and bromolysergic acid diethylamide and by Haverback, Stubrin & Dyce (1965), who administered 5-hydroxytryptamine and 1-(τ)-methylhistamine to dogs with a total stomach gastric fistula. Both groups of authors described an augmentation of the histamine- and gastrin-stimulated acid secretion by the previously mentioned agents. But histamine methyltransferase is absent in the gastric mucosa of rats (Brown *et al.*, 1959; Kim, Backus, Harris & Rourke, 1969), and Haverback *et al.* (1965) used very small doses of the drugs (0.5-1.0 $\mu g\ kg^{-1}\ min^{-1}$), which are far below that of ID_{50} for an inhibition of histamine methyltransferase *in vitro* (Brown *et al.*, 1959). Thus it is not clear by which mechanism the drugs mentioned above enhanced gastric acid secretion.

The question whether the enhancement of the histamine-stimulated acid secretion by amodiaquine was the result of a local inhibition of histamine methyltransferase or of a general delay in histamine inactivation can partly be answered by determining the elimination-rate of exogenously administered histamine from dog's plasma after the injection of amodiaquine. Applied in doses of 2.5 and 5.0 mg/kg this compound did not protract histamine elimination from the plasma (Lorenz *et al.*, 1974). Furthermore, amodiaquine did not release histamine (Lorenz *et al.*, 1974). Thus, amodiaquine seemed to enhance gastric acid secretion by inhibiting histamine methyltransferase in the glandular part of the canine mucosa, but not by influencing the concentrations of histamine in the systemic circulation.

However, explanations for the effect of amodiaquine on gastric secretion other than inhibition of gastric histamine methyltransferase, have to be considered. A direct action on the parietal cell due to calcium mobilization or to influences on the system of cyclic nucleotides can largely be excluded since amodiaquine does not cause acid secretion when given alone (Troidl *et al.*, 1973). Vagal stimulation by amodiaquine seems unlikely for the same reason because prostigmine and carbachol elicited a considerable gastric secretion in the same animals in which amodiaquine was completely ineffective (Troidl *et al.*, 1973).

We wish to thank Dr K Goecke and Dr H. Rohde for their skilful assistance. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Lo 199/3)

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(Received March 29, 1974.
Revised February 17, 1975.)

Br. J. Pharmac. (1975), 55, 329-333

ATYPICAL TRYPTAMINE RECEPTORS IN SHEEP PULMONARY VEIN

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1 Both the pulmonary artery and vein of the sheep contracted dose-dependently to histamine, carbamoylcholine, prostaglandin $F_{2\alpha}$, noradrenaline and bradykinin and relaxed in the presence of isoprenaline or prostaglandin E_1.

2 The effect of 5-hydroxytryptamine (5-HT) on the artery was consistently to produce dose-dependent contractions without tachyphylaxis. The effect on the vein was biphasic. 5-HT 5×10^{-10} to 5×10^{-8} M relaxed the partially constricted vein. 5-HT 10^{-7} to 10^{-6} M caused brief venoconstriction followed by relaxation. 5-HT $> 10^{-6}$ M caused dose-related contraction of the vein.

3 Methysergide effectively blocked the contractile response of the artery to 5-HT, but only weakly inhibited the contractions of the vein (dose-ratio <20).

4 Each of ten antagonists tested failed to inhibit the 5-HT-induced relaxation of the vein. Sheep pulmonary vein possesses tryptamine receptors which mediate relaxation and which are not of the classical M- or D-type. These receptors appear not to be involved directly or indirectly with responses to acetylcholine, catecholamines, histamine or prostaglandins.

Introduction

Dietary tryptophan is reported to be a principal aetiological factor in the development of a form of acute inflammatory reaction of bovine lung characterized by oedema, congestion, bronchospasm and emphysema: known as acute bovine pulmonary emphysema (Dickinson, Spencer & Goreham, 1967; Blake & Thomas, 1971). The suggested pathogenesis is that tryptamine analogues are synthesized from tryptophan in the digestive tract, probably in the rumen contents (Schatzmann & Gerber, 1972). 5-Hydroxytryptamine (5-HT) (Eyre, 1972, Schatzmann & Gerber, 1972), tryptamine (Schatzmann & Gerber, 1972) and 3-methylindole (Carlson, Yokoyama & Dickinson, 1972) have been related to bovine pulmonary emphysema. Carlson, Dyer & Johnson (1968) experimentally reproduced the disease in cattle by feeding DL-tryptophan to cattle, whereas this amino acid led to no observable clinical or pathological effects in sheep.

It was important to screen a number of tryptamine antagonists in order to suggest suitable therapy for the disease in cattle (to be published elsewhere). It was also of pharmacological interest to study the actions of tryptamine analogues and antagonists on sheep pulmonary blood vessels as a possible means of explaining the peculiar species difference.

Methods

Lungs were obtained from healthy sheep of mixed breed, age and of either sex which were killed with pentobarbitone sodium. The principal pulmonary artery and vein were dissected out, cut spirally and mounted individually in similar organ-baths of 20 or 30 ml volume in Krebs-Henseleit solution, gassed with 5% CO_2 in O_2 at 35° C as previously described (Eyre, 1971). Movements of the mucle strips were recorded with isotonic myograph transducers and an E & M Model 4 Physiograph. All tissues were exposed at random to at least three concentrations of agonists for 2 min or until maximum response, every 10-15 minutes. Three-point dose-response curves to each of two or three predetermined agonists were established in each strip, 5-HT being included in every case. Before injection of 5-HT, the muscle strip was sub-maximally contracted (approximately 50%) by histamine or carbamoylcholine (Eyre, 1973).

Antagonists were applied when the agonist dose-effect relationship was stable. A specific antagonist was added to each bath and the drug left in contact with the tissue for at least 30 min before agonist dose-response curves were re-established. The activity of an antagonist was estimated by measuring the dose-ratio of each agonist, i.e. the ratio of doses of agonist which give equal responses in the presence and absence of

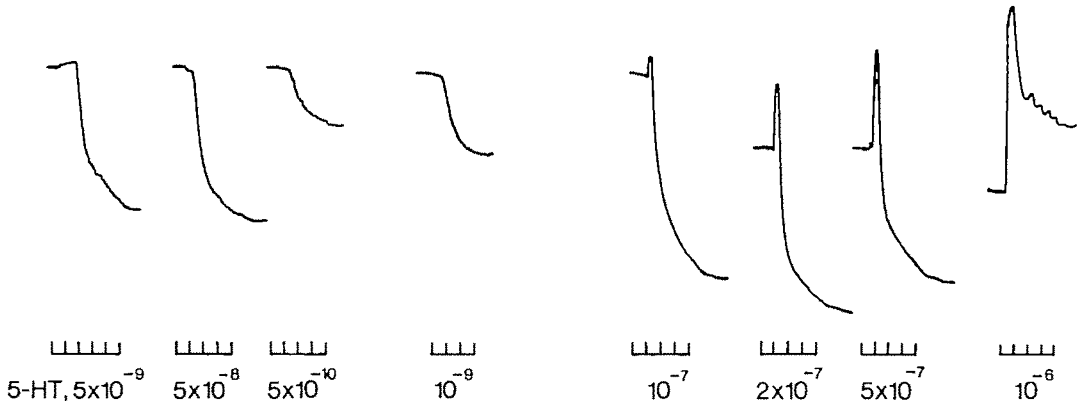

Figure 1 Isolated helical strip of sheep pulmonary vein in 20 ml Krebs-Henseleit solution gassed with 5% CO_2 in O_2 at 35° C. Preparation is partially contracted (approximately 50%) by histamine prior to each addition of 5-hydroxytryptamine (conc. range $5 \times 10^{-10} - 10^{-6}$ M). Marker shows injection times and 0.5 minutes.

antagonist (Gaddum, Hameed, Hathway & Stephens, 1955).

Drugs

The following drugs were used: histamine acid phosphate, 5-hydroxytryptamine creatinine sulphate, carbamoylcholine chloride, noradrenaline bitartrate, isoprenaline hydrochloride, bradykinin triacetate, tryptamine hydrochloride, methysergide bimaleate, dibenamine hydrochloride, propranolol hydrochloride, atropine sulphate, morphine sulphate, mepyramine maleate, burimamide, indomethacin, sodium meclofenamate and phloretin phosphate dimer.

Results

5-HT (5×10^{-10} to 5×10^{-8} M) induced dose-dependent relaxation of the sheep pulmonary vein which had been partially contracted by histamine (10^{-8} to 10^{-7} M). 5-HT concentrations between approximately 10^{-7} and 10^{-6} M caused brief contractions rapidly followed by relaxation (net response = relaxation) and concentrations of 5-HT > 10^{-6} M caused only contractile responses in the partially contracted vein (Figures 1 and 2).

Tryptamine caused qualitatively similar effects to 5-HT (four observations each drug) but was quantitatively weaker (equipotent molar ratios: 5-HT = 1; tryptamine = 155).

Methysergide (5×10^{-7} M) inhibited all contractile effects of 5-HT irrespective of the dose of 5-HT used. However, methysergide >5×10^{-6} M itself contracted the vein strip, which precluded measurements using greater concentrations of the antagonist. Dibenamine (10^{-8} to 10^{-6} M) did not inhibit 5-HT-induced contractions of the vein.

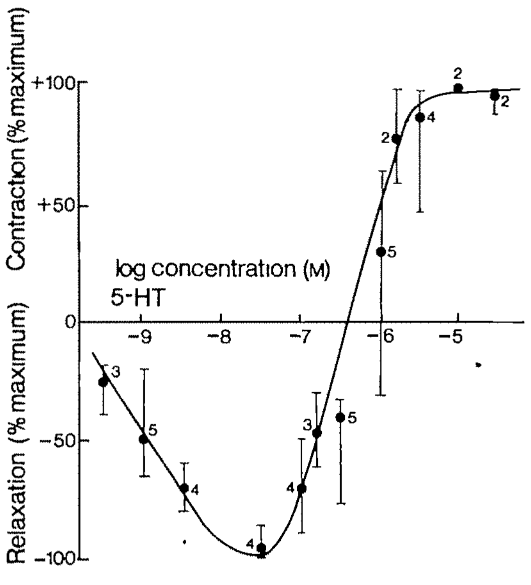

Figure 2 Log concentration (M) response curve (% maximum) to 5-hydroxytryptamine in isolated helical strips of sheep pulmonary vein in 20 ml Krebs-Henseleit solution gassed with 5% CO_2 in O_2 at 35° C. Numbers on graph refer to numbers of observations. Vertical bars represent ranges of values.

Ten antagonists were used in an attempt to block 5-HT-induced relaxation. No antagonist tested had any measurable inhibitory action on the relaxant response 5-HT. Specific antagonists used, selectively blocked their complementary agonists (Table 1). For example, propranolol inhibited the

Table 1 Antagonism of histamine, carbachol, prostaglandins E_1 (PGE_1) and $F_{2\alpha}$ ($PGF_{2\alpha}$), bradykinin, noradrenaline, isoprenaline and 5-hydroxytryptamine (5-HT) on isolated helical smooth muscle strip of sheep pulmonary vein

		Dose ratios of agonists*							
Antagonist	*Conc. (M)*	*Histamine*	*Carbachol*	*PGE_1*	*$PGF_{2\alpha}$*	*Bradykinin*	*Noradrenaline*	*Isoprenaline*	*5-HT*
Methysergide	10^{-7}	—	—	—	—	—	—	—	1.0(2)
	2×10^{-6}	2.2(2)	—	—	—	—	1.5(2)	—	1.5(4)
Dibenamine	10^{-7}	—	—	—	—	—	20.0(2)	—	1.2(2)
	2×10^{-6}	1.8(2)	—	—	—	—	31.0(2)	—	1.4(2)
Propranolol	10^{-7}	—	—	1.5(2)	—	—	—	42.0(2)	1.1(2)
	2×10^{-6}	3.0(2)	2.5(2)	1.6(3)	—	—	—	75.0(3)	1.5(3)
Atropine	2×10^{-7}	—	55.0(3)	—	—	—	—	—	1.2(3)
	2×10^{-6}	2.5(2)	92.0(2)	—	—	—	—	—	1.0(2)
Morphine	2×10^{-7}	1.5(3)	—	—	—	—	—	—	1.1(4)
	5×10^{-6}	2.1(2)	3.0(2)	—	—	—	—	—	1.0(3)
Mepyramine	10^{-8}	151.0(3)	2.5(2)	—	—	—	—	—	1.0(3)
	10^{-7}	510.0(3)	—	—	—	5.5(2)	—	—	1.0(3)
Burimamide	10^{-6}	1.0(3)	—	—	—	—	—	—	0.9(3)
	5×10^{-5}	0.9(3)	—	—	—	—	—	—	1.0(3)
Indomethacin	5×10^{-7}	—	—	1.5(3)	—	—	—	—	1.5(3)
	5×10^{-5}	—	—	1.5(2)	1.0(2)	—	—	—	1.5(2)
Meclofenamate-Na	10^{-7}	—	6.1(2)	5.0(2)	2.0(2)	5.5(2)	—	—	1.1(2)
Phloretin phosphate	3×10^{-5}	1.5(2)	—	18.5(2)	—	—	—	—	1.2(2)
dimer	1.5×10^{-4}	1.4(2)	—	25.1(2)	6.5(2)	—	—	—	1.4(2)

* Dose-ratio is the ratio of equiactive doses (concentrations) of agonist in the presence and absence of antagonist. Each figure is a mean and numbers of observations are in parentheses.

effect of isoprenaline; atropine the effects of acetylcholine; mepyramine the actions of histamine and phloretin phosphate dimer inhibited prostaglandin E_1 in the same preparation, without blocking 5-HT. It was interesting that indomethacin itself relaxed the pulmonary vein (possibly due to inhibition of endogenous prostaglandin production) but when the vessel was partially contracted again by histamine or acetylcholine the superimposed relaxant effect of 5-HT was unimpaired. The three classical antagonists of 5-HT (methysergide, dibenamine and morphine) did not inhibit the relaxant actions of 5-HT in this tissue. Drugs which are known to block prostaglandin synthesis (indomethacin and meclofenamate), or which block prostaglandin receptors (phloretin phosphate) did not impair the relaxant effect of 5-HT.

By contrast, the sheep pulmonary artery was conistently contracted by 5-HT (threshold dose 10^{-8} to 10^{-6} M) dose-dependently and without tachyphylaxis. The arterial response was specifically antagonized by methysergide (dose-ratio = 120 at 10^{-6} M) but not by morphine (up to 10^{-5} M) nor by dibenamine (up to 5×10^{-6} M).

Discussion

Two kinds of tryptamine receptors have been classified according to antagonists. Gaddum & Piccarelli (1957) suggested there were neuronal receptors (possibly in ganglia or nerve fibres) which are blocked by morphine and atropine, the so-called M-receptors. A second type of receptor was postulated in smooth muscle, the D-receptors, which are blocked by dibenzyline and lysergic acid diethylamide (LSD) derivatives.

From the data presented here it appears that the sheep pulmonary artery carries only D-receptors (muscular), blocked by methysergide, though unexpectedly not blocked by dibenamine. Contractile actions of tryptamine analogues on sheep pulmonary veins may also be mediated by D-receptors, whereas the 5-HT-induced relaxant effect appears to be mediated via a third type of tryptamine receptor which may not be pharmacologically related to the classical M- and D-types (on the basis of antagonists at least), nor involved directly or indirectly with responses to acetylcholine, catecholamines, histamine or prostaglandins.

Wurzel (1966) and Bakhle & Smith (1974) described tryptamine receptors in rabbit aorta and rat pulmonary artery respectively which do not fit into the original Gaddum & Picarelli classification. Receptors of the rabbit aorta were inhibited non-specifically by dibenzyline and dichloriso-proterenol, but not by morphine or LSD. Receptors of the rat pulmonary artery were blocked both by morphine and methysergide. As far as can be ascertained the present paper may be the first to describe pulmonary vasodilatation mediated by tryptamine receptors which are refractory to blockade. As Wurzel (1966) pointed out, the Gaddum-Picarelli convention for tryptamine receptors leaves much to be desired. Most autacoid receptors have been classified by both specific agonists and specific antagonists; usually on the basis of a structure-action relationship. Gaddum & Picarelli's 'D and M' classification is based neither on structurally-related agonists nor specific antagonists. The so-called atypical nature of certain tryptamine receptors (Wurzel, 1966; Bakhle & Smith, 1974; and this paper) may be more imagined than real. More extensive studies of tryptamine receptors in many tissues are indicated. This may then permit a re-appraisal of the classification.

The pulmonary vein is some 10 to 100 times more sensitive to 5-HT than the artery in both cow and sheep (Eyre, 1970; and this paper). The response of bovine pulmonary blood vessels to tryptamine analogues is purely contractile. It is probable that pulmonary venospasm and increased capillary permeability contribute to the tryptamine-induced inflammatory response of bovine lung. On the other hand, venodilatation is the most sensitive pulmonary vascular tryptaminergic response of sheep and it therefore seems reasonable to suggest that in this species the naturally-occurring tryptamine analogues may be in a sense counterinflammatory. It is interesting to think of this as a possible evolutionary advantage that sheep have acquired over their bovine relatives.

The work was made possible by grants from the Ontario Ministry of Agriculture and Food and Grant A5937 of the National Research Council of Canada. The author is grateful to Mr T.R. Deline for technical assistance and also to the following companies for generous gifts of drugs:– Ayerst of Montreal, Canada, for propranolol; A.B. Leo of Halsingborg, Sweden, for PP dimer; Parke Davis & Co., Ann Arbor, Michigan, U.S.A., for sodium meclofenamate; Poulenc of Montreal, Canada, for mepyramine; Sandoz of Basle, Switzerland, for methysergide; Merck, Sharp & Dohme, Rahway, N.J., U.S.A., for indomethacin and Smith, Kline & French of Welwyn Garden City, England, for burimamide.

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(Received January 16, 1975.
Revised June 27, 1975.)

Br. J. Pharmac. (1975), 55, 335-341

THE PHARMACOKINETICS OF PYRIDOSTIGMINE AND 3-HYDROXY-*N*-METHYLPYRIDINIUM IN THE RAT: DOSE-DEPENDENT EFFECTS AFTER PORTAL VEIN ADMINISTRATION

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1 The elimination kinetics of [^{14}C]-pyridostigmine iodide and [^{14}C-methyl]-3-hydroxypyridinium bromide (3-OH NMP) have been studied in the rat.

2 For pyridostigmine, at a given dose level, the fraction of the dose eliminated unchanged was reduced and the metabolite fraction was increased after portal vein administration when compared to jugular vein administration. This indicates that pyridostigmine is subject to metabolism during the first passage through the liver.

3 When doses of pyridostigmine 1.25 μmol/kg and higher were injected via the portal vein, the proportion excreted in urine as unchanged drug remained constant; in contrast, the percentage of the dose eliminated as the metabolite was significantly reduced. This indicates that a dose-dependent process is involved in the urinary excretion of 3-OH NMP.

4 This conclusion was supported by studies involving the portal and systemic venous injection of 3-OH NMP at different dose levels. After 4 h, approximately 85% of the lowest dose was eliminated unchanged in urine; in contrast, only 63% of the higher dose was excreted during this period. The proportion of the dose eliminated in urine was not related to the route of administration.

5 After the injection of pyridostigmine into the jugular vein, the initial rate of drug excretion fell rapidly for approximately 10 min; in contrast, after injection into the portal vein, the rate of excretion of the drug rose to a maximum at 30 minutes. This suggests that the hepatoportal system behaves as a distinct region during the distribution of this drug.

Introduction

Previous pharmacokinetic studies with pyridostigmine have been mainly concerned with its metabolism and renal excretion. In the rat and in man, the quaternary amine is mainly metabolized to 3-hydroxy-*N*-methylpyridinium (3-OH NMP), and both unchanged pyridostigmine and the metabolite are subsequently excreted in urine (Birtley, Roberts, Thomas & Wilson, 1966; Kornfeld, Samuels, Wolf & Osserman, 1970, Somani Roberts & Wilson, 1972). Small amounts of other unidentified metabolites have also been isolated from human urine (Kornfeld *et al.*, 1970; Somani *et al.*, 1972).

The present paper is concerned with the pharmacokinetics of pyridostigmine and 3-OH NMP in the rat. When drugs are cleared from plasma by first-order kinetics, both the biological half-life and the fractional composition of excretion products are independent of the dose. Alternatively, drugs may be eliminated by dose-dependent processes; in these conditions deviations from apparent first-order kinetics may be detected by changes in the fractional composition of excretion products, as long as renal clearance is constant (Levy, 1968). Recent evidence suggests that many drugs may be eliminated in this manner (Wagner, 1973). Pharmacokinetic studies of the quaternary amines neostigmine and 3-hydroxyphenyltrimethylammonium have demonstrated that both these compounds are eliminated by dose-dependent processes and subject to 'first pass' metabolic effects by the liver (Barber & Bourne, 1974).

The aim of this investigation was to characterize the elimination kinetics of pyridostigmine and 3-OH NMP in terms of either first-order or dose-dependent processes. The effect of the dose and the route of administration of both these drugs on the fractional composition of excretion products in urine has been studied.

Part of this work has already been communicated to the British Pharmacological Society (Barber & Bourne, 1973b).

Methods

Experimental procedure

Male Wistar rats (body weight· 250-350 g) were anaesthetized with ethyl carbamate (14% w/v in distilled water; 1.4 g/kg, i.p.) and the trachea was cannulated. A jugular vein and both ureters were also cannulated with polyethylene tubing (Portex pp 50). Mannitol (6% w/v in 0.9% w/v NaCl solution; 75 µl/min) was continuously infused via the jugular vein to maintain a constant diuresis (Barber & Bourne, 1971).

In experiments in which drugs were administered via the portal vein, a 22 gauge needle attached to a length of fine polyethylene tubing (Portex pp 10) was inserted into the portal vein near the hilum of the liver (Back & Calvey, 1972). The other end of the polyethylene tubing was attached to a needle and syringe.

Pharmacokinetic studies

[^{14}C]-pyridostigmine iodide (N-[^{14}C methyl]-3-(*N,N*-dimethyl-carbamato) pyridinium iodide (The Radiochemical Centre, Amersham; sp. act. = 16.1 mCi/mmol) was administered alone or with non-labelled compound. The drug was rapidly injected via the jugular vein (dose: 0.10 or 0.63 µmol/kg) or via the portal vein (dose: 0 10, 0.63, 1.25, 3.00, or 6.0 µmol/kg). Urine was collected in 10, 15, 20 or 30 min periods for up to 4 hours.

[^{14}C]-3-OH NMP (N-[^{14}C-methyl]-3-hydroxypyridinium bromide; Roche Products Limited; sp. act. = 3.9 mCi/mmol) was rapidly administered via the jugular vein (dose: 0.10, 1.64 or 16.4 µmol/kg) or via the portal vein (dose: 0.10 or 1.64 µmol/kg). Samples of urine were collected at 15, 30, 45, 60, 90, 120, 180 and 240 minutes. In all experiments, urine volume was indirectly determined by dividing the weight of each sample by its relative density (assuming that d = 1.000).

The lower dose used in this study is comparable with that routinely used in the treatment of myasthenia gravis; amounts in the higher doses are similar to those sometimes used to reverse the effects of tubocurarine in anaesthetic practice.

Liquid scintillation counting and assay procedures

Samples of urine (50 µl) were analysed for total radioactivity by liquid scintillation counting, using 10 ml of a homogenous Triton-X-100 solution (6 g butyl P.B.D. (2(4′-*t*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole) in 667 ml toluene, 333 ml Triton-X-100 and 75 ml water) (Barber & Bourne, 1973a). All samples were counted in a Nuclear Chicago Unilux II scintillation spectrometer. Quench corrections were made by the channels ratio method.

Samples of urine were usually analysed for pyridostigmine and its metabolites by electrophoresis in borate buffer (0.1 M, pH = 9.2, 2 h, 300 V) (Somani *et al.*, 1972). The paper electrophoretogram was counted on a 4π scanner (Tracerlab) to determine the relative proportions of the unchanged drug and its metabolites. Most urine samples were resolved by ion exchange chromatography using Amberlite IRC-50 resin. The eluate was then assayed for the parent drug and its metabolites by liquid scintillation spectrometry.

Results

After injection of pyridostigmine via the jugular vein, an average of 52% was eliminated unchanged in urine in 4 hours. A further 22% was excreted as 3-OH NMP during this time. The proportion of the dose eliminated in urine as pyridostigmine and its metabolite was independent of the dose administered. When the same doses of pyridostigmine were injected into the portal vein, an average of 47% was excreted as the unchanged drug in 4 hours. An additional 40% was eliminated as the metabolite during this period (Table 1).

When doses of pyridostigmine 1.25 µmol/kg and higher were injected via the portal vein, the proportion excreted in urine as unchanged drug remained constant; in contrast, the percentage of the dose eliminated as the metabolite was significantly reduced (Table 2). Thus these results show that a dose-dependent process is involved in the urinary excretion of 3-OH NMP.

This conclusion was supported by studies involving the portal and systemic venous injection of 3-OH NMP at different dose levels.

After 4 h, approximately 85% of the lowest dose was eliminated unchanged in urine; in contrast, only 63% of the higher doses was excreted during this period. The proportion of the dose eliminated in urine was not related to the route of administration (Table 3).

After intravenous injection of pyridostigmine, the rate of excretion of the unchanged drug (expressed as the percentage of the dose eliminated per min) was determined for each urinary collection period. This value was plotted semilogarithmically against the time at the mid-point

Table 1 Fraction of the dose eliminated in urine after administration of pyridostigmine to rats via the jugular or portal vein at the same dose level

		% dose eliminated	
Dose and Route	*No. of animals*	*0-2 h*	*0-4 h*
Via jugular vein *0.10 μmol/kg*			
Pyridostigmine	4	48 ± 2*	53 ± 1**
Metabolite		13 ± 2†	22 ± 3††
0.63 μmol/kg			
Pyridostigmine	4	43 ± 4	51 ± 3
Metabolite		10 ± 1	21 ± 3
Via portal vein *0.10 μmol/kg*			
Pyridostigmine	3	40 ± 3*	43 ± 3**
Metabolite		27 ± 3†	40 ± 2††
0.63 μmol/kg			
Pyridostigmine	2	38 54	42 57
Metabolite		23 35	38 44

Results are mean ± s.e. mean.
* and ** significantly different from each other ($P = 0.05$) † and †† significantly different from each other ($P = 0.01$).
Probability was determined by Student's *t* test.

Table 2 Fraction of the dose eliminated in urine after administration of pyridostigmine to rats via the portal vein. Doses are greater than 1 μmol/kg.

Time (min)	*1.25 μmol/kg (a)*	*3.00 μmol/kg (b)*	*6.00 μmol/kg (c)*	P
Pyridostigmine				
60	21 ± 2	27 ± 6	21 ± 3	a to b > 0.3** a to c > 0.9**
100	29 ± 2	36 ± 5	28 ± 3	a to b > 0.2** a to c > 0.8**
130	33 ± 5	41 ± 4	34 ± 2	a to b > 0.2* a to c > 0.8*
Metabolite				
60	11 ± 2	7 ± 1	6 ± 1	a to b = 0.05** a to c = 0.05**
100	23 ± 2	15 ± 1	14 ± 3	a to b < 0.02** a to c < 0.05**
140	34 ± 2	22 ± 1	22 ± 4	a to b < 0.01* a to c < 0.05*

Results are mean ± s.e. mean.
Probability was determined by Student's *t* test.
* d.f. = 4; ** d.f. = 6.

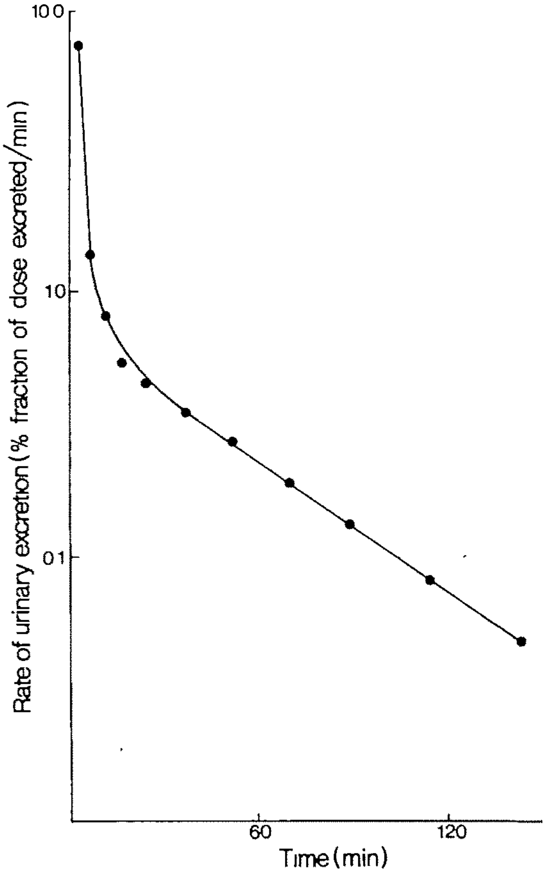


Figure 1 An example of the rate of urinary excretion of pyridostigmine after jugular vein administration. Dose 0.63 μmol/kg.

of each urine collection, after allowing a delay of 4 min between filtration at the glomerulus and the appearance of the drug in the collecting vessel (Bourne & Barber, 1972). After the injection of pyridostigmine into the jugular vein, the initial rate of drug excretion fell rapidly for approximately 10 min; this phase was followed by a less rapid logarithmic decline in the rate of urinary elimination (Figure 1). In contrast, after injection of pyridostigmine into the portal vein, the rate of excretion of the drug rose to a maximum at 30 min, before declining in a logarithmic manner (Figure 2) Thus the urinary excretion profile of pyridostigmine is clearly dependent on the route of administration of the drug (cp. Figures 1 & 2). Similar differences were not observed when 3-OH NMP was injected into the systemic and the portal circulation. In these conditions, the rate of urinary excretion of 3-OH NMP was maximal initially and then fell exponentially, irrespective of the route of administration. Thus, the results resembled the urinary profile observed after injection of pyridostigmine into the systemic venous system (Figure 1).

Paper electrophoresis and ion exchange chromatography (Somani *et al.*, 1972) failed to provide any evidence for the subsequent metabolism of 3-OH NMP. A single peak of radioactivity with an electrophoretic mobility similar to authentic 3-OH NMP was identified. The duration of all the experiments was relatively short, and total recovery of the radioactivity administered was not achieved. Nevertheless, it is considered that the results adequately reflect the pharmacokinetics of pyridostigmine and 3-OH NMP, since the relative

Table 3 Fraction of the dose eliminated in urine after administration of 3-hydroxy-*N*-methyl pyridinium to rats via the jugular or portal vein

Dose and route	*No. of animals*	*% dose eliminated* 0-2 h	0-4 h
0.10 μmol/kg			
via jugular vein	7	73 ± 4	84 ± 3
via portal vein	7	71 ± 1	87 ± 2
1.64 μmol/kg			
via jugular vein	7	62 ± 8	63 ± 8
via portal vein	7	58 ± 3	64 ± 3
16.4 μmol/kg			
via jugular vein	7	54 ± 6	63 ± 5

Results are mean ± s.e. mean.

The fraction eliminated at the higher doses is significantly lower (*P* < 0.01; Student's *t* test) than the fraction eliminated after the dose of 0.1 μmol/kg by either route of administration.

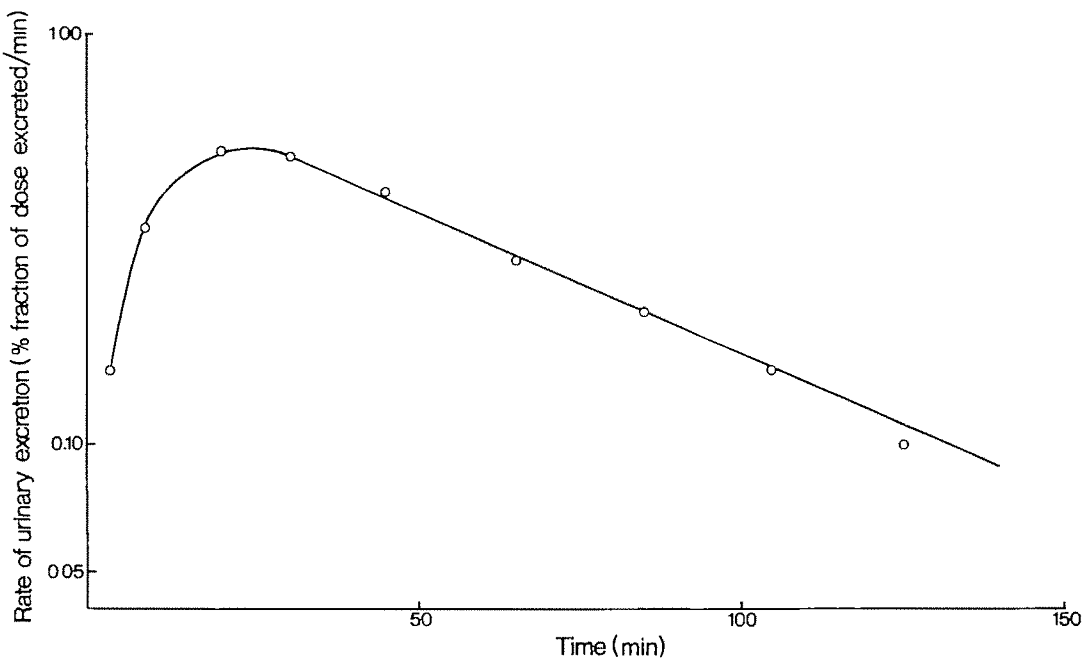


Figure 2 An example of the rate of urinary excretion of pyridostigmine after portal vein administration Dose 6.0 μmol/kg.

proportions of both compounds in urine invariably approached an asymptote.

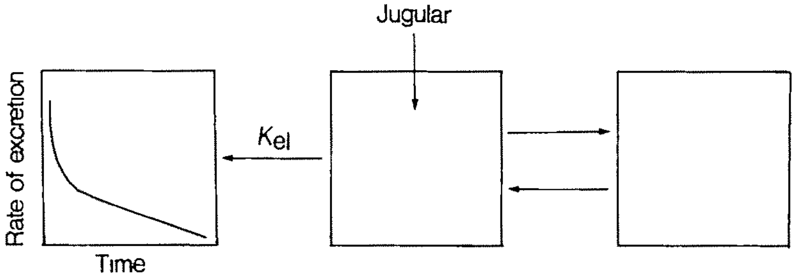
Discussion

The present experiments suggest that there are qualitative differences between the elimination kinetics of pyridostigmine and 3 OH NMP. Thus, after injection of pyridostigmine into the portal vein, the proportion eliminated as unchanged drug was independent of the dose in the range studied. In contrast, the urinary excretion of 3-OH NMP was dose-dependent; with small doses of pyridostigmine, a larger fraction was eliminated as the metabolite, 3-OH NMP. After large doses of pyridostigmine, enzymatic hydrolysis to 3-OH NMP was not rate-limiting, since the decrease in the fraction eliminated as the metabolite was not accompanied by an increase in the proportion excreted as the parent drug. This conclusion was consistent with the results obtained after administration of 3-OH NMP via the jugular or the portal vein. In these conditions, the quantitative excretion of the drug in urine was dependent on the dose; after high doses of 3-OH NMP, the proportion eliminated was significantly decreased. These results may reflect the excretion of 3-OH NMP by carrier transport in the proximal renal tubule. Many quaternary amines are eliminated in urine by a tubular transport system with a finite and saturable secretory capacity. The present experimental findings are consistent with the excretion of 3-OH NMP by renal tubular transport, with a secretory capacity that is saturated within the dose range of 0.1-1.64 μmol/kg.

When a drug is eliminated in urine from the region in which it is first distributed, then a semilogarithmic plot of urinary excretion rate with time should give, assuming first order rate processes, a characteristic biexponential curve with a maximum at zero time (Janku & Krebs, 1971). However, if the drug is first distributed into a body compartment from which it is not eliminated, then the rate of excretion curve with time should increase to a maximum before falling, as the drug moves from the initial region of distribution to the compartment from which elimination takes place (see Figure 3).

Pharmacokinetic studies with other quaternary amines (Barber & Bourne, 1974) have not established unequivocally whether these compounds are eliminated from the region in which they are first distributed. On the one hand, both neostigmine

Simultaneous distribution and elimination after jugular vein administration



Distribution within a compartment before elimination after portal vein administration

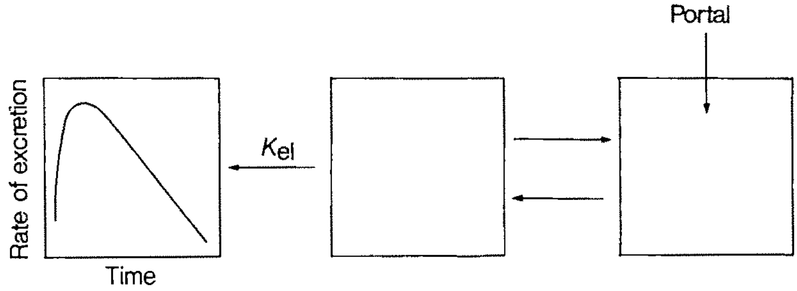


Figure 3

and 3-hydroxy-phenyltrimethylammonium (3-OH PTMA) are subject to a first pass effect. This strongly suggests that the hepatoportal system acts as a distinct region for the distribution of these compounds. On the other hand, the character of the urinary excretion rate plots after portal vein administration and jugular vein administration was closely similar, indicating that elimination takes place from the region in which the drug is first distributed. If the hepatoportal system is a separate region of distribution for these compounds, then the pattern of the rate of excretion should be different after portal and jugular venous injection (Figure 3). The failure to observe this phenomenon may be due to the rapid and extensive metabolism of neostigmine and 3-OH PTMA. Less than 8% of a dose of 3-OH PTMA, and only 5% of a dose of neostigmine are eliminated unchanged, and it may not be possible to detect the distributive phase of such a small fraction of the dose.

Different conditions clearly apply to the distribution of pyridostigmine. After the injection of the drug into the systemic circulation, the initial rate of drug excretion fell rapidly, and was followed by a less rapid linear decline in the rate of urinary elimination. In contrast, after intraportal injection of the drug, the rate of excretion rose to a maximum at 30 min before falling. Thus, the hepatoportal system behaves as a distinct region during the distribution of the drug (Figures 2 & 3).

The fraction of the dose eliminated as pyridostigmine was reduced and the metabolite fraction greatly increased after portal vein administration of a given dose when compared to jugular vein administration of the same dose (Table 1); thus pyridostigmine is subject to metabolism during the first passage through the liver. These results are similar to those reported for the related quaternary amines neostigmine and its metabolite 3-OH PTMA (Barber & Bourne, 1974). It is also possible that the lower fraction of the metabolite excreted after jugular venous administration may be indicative of competition for a renal secretory site by the initial high concentration of pyridostigmine attained by this route of administration.

Differences in the excretion pattern of neostigmine and pyridostigmine may be related to the time of onset of their pharmacological effects. After portal venous administration of neostigmine and pyridostigmine in doses which are equipotent at the time of their maximal plasma concentration, the latent period before the onset of their actions will differ. Thus, neostigmine will immediately reach its maximum level in plasma, while the concentration of pyridostigmine will slowly rise to a maximum at 30 minutes. In the rat, the onset of muscle fasciculation is rapid after intraportal neostigmine, with pyridostigmine a slower onset of action is observed. Similar effects may occur after oral administration, irrespective of any differences in intestinal absorption.

We would like to thank Dr R.F. Long, of Roche Products Limited, Welwyn Garden City, Herts, for the gift of 3-OH NMP. We would also like to thank Professor A. Breckenridge for his advice in the writing of this manuscript. The financial support of the Muscular Dystrophy Group of Great Britain is acknowledged.

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(Received February 26, 1975.
Revised May 21, 1975.)

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EVIDENCE FOR NORADRENALINE AND ADRENALINE AS SYMPATHETIC TRANSMITTERS IN THE CHICKEN

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1 The concentrations of noradrenaline and adrenaline in various organs, arterial plasma and venous outflow from isolated hearts of adult chickens have been determined.

2 The relative adrenaline concentrations (percentage of the sum of noradrenaline and adrenaline) in the heart (33%), spleen (16%) and brain (26%) were higher than those found in mammalian organs. Chemical sympathectomy by pretreatment with 6-hydroxydopamine caused a decrease of the noradrenaline and adrenaline concentrations in the heart to 20 and 23% and in the spleen to 16 and 29%, respectively.

3 Stimulation of the right sympathetic nerves, infusion of tyramine or infusion of a modified Tyrode solution containing 108 mM K^+ and 44 mM Na^+ caused an output of both noradrenaline and adrenaline into the perfusate of isolated hearts. The relative adrenaline concentration in the perfusate (20-28%) was not significantly different from the relative adrenaline concentration remaining in these hearts (19-22%). In the individual experiments, the noradrenaline. adrenaline ratios of the stimulation perfusates were positively correlated with the ratios found in the hearts.

4 The effects of noradrenaline and adrenaline on cardiac rate and tension development were studied in spontaneously beating right atria and electrically driven left atria, respectively. In addition, the arterial pressure rise in response to noradrenaline or adrenaline was measured in chickens. It was found that the cardio-vascular potency of noradrenaline, as reflected by the increases in heart rate, cardiac tension development and arterial blood pressure, was not significantly different from that of adrenaline.

5 It is concluded that, in the chicken heart and spleen, both noradrenaline and adrenaline act as sympathetic neurotransmitters.

Introduction

It has been shown that both noradrenaline and adrenaline are present in the brain and peripheral tissues of chicks and chickens (review by Holzbauer & Sharman, 1972). The concentrations of adrenaline found in these tissues are higher than in the corresponding tissues of mammals. Thus the question of whether noradrenaline or adrenaline is the sympathetic transmitter in the chicken has been raised. Callingham & Cass (1966) speculating from indirect evidence, suggested that adrenaline might fulfil this function and that noradrenaline acts only as a precursor. However, Sturkie & Poorvin (1973) obtained direct evidence that noradrenaline is a sympathetic transmitter by showing that sympathetic nerve stimulation caused a release of noradrenaline from the isolated heart of the chicken. As the authors did not detect a release of adrenaline following nerve stimulation, the fate and function of adrenaline present in sympathetically innervated organs remained obscure. Interestingly the same controversy exists amongst histologists using fluorescent histochemical techniques to distinguish the roles of noradrenaline and adrenaline in adrenergic transmission (Bennett & Malmfors, 1970).

Preliminary results of the present work were presented at the meeting of the Deutsche Pharmakologische Gesellschaft (Lindmar &

DeSantis, 1974) and the Second Congress of the Hungarian Pharmacological Society (Löffelholz & Lindmar, 1974).

Methods

Collection of blood and isolation of organs

The chickens used for the experiments were white adult HNL (Heisdorf-Nelson-Lohmann) hens 3-19 months old weighing 1.0-1.8 kg. The hens were anaesthetized with sodium hexobarbitone (40 mg/kg, i.v. into the brachial vein), and the thoracic cage opened so as to reveal the heart. A cannula was inserted into the left ventricle and one blood sample of approximately 30 ml or three samples of 10 ml each were collected in tubes containing heparin (250 i.u. dissolved in 0.2 ml 0.9% w/v NaCl solution (saline)). The exsanguination procedure lasted approximately 1 minute. The heart, spleen, adrenal glands and brain were then removed for estimation of noradrenaline and adrenaline. Cerebellum and cerebral hemispheres were dissected from the brains, leaving the thalamus, hypothalamus, midbrain and medulla behind.

Perfusion of the heart

In the release experiments, the isolated hearts were perfused by the Langendorff technique with Tyrode solution of the following composition (mM): Na^+ 149.3, K^+ 2.7, Ca^{++} 1.8, Mg^{++} 1.05, Cl^- 145.4, HCO_3^- 11.9, $H_2PO_4^-$ 0.4, (+)-glucose 5.6. The solution contained ascorbic acid 10 mg/l and was maintained at 34° C. This solution was gassed with a mixture of 95% O_2 and 5% CO_2. The perfusion apparatus which is described in detail elsewhere (Kuschinsky, Lindmar & Wollert, 1974) allowed a rapid change from one perfusion medium to another.

The hearts were first perfused with a constant pressure (600 mmH_2O) to detect accidental coronary occlusions (e.g. blood clot, gas bubble). The flow rate was 35 ± 2 ml/min ($n = 34$) at this pressure. Thereafter the hearts were perfused with a constant volume (24-25 ml/min) using a Desaga peristaltic pump. The heart rate was measured by counting the contractions in a 15 s period.

Sympathetic nerve stimulation

In some experiments the right sympathetic nerves were exposed according to Tummons & Sturkie (1969) and isolated up to the sympathetic chain. Then the hearts with the nerves attached were removed and perfused as described above. The nerves were stimulated with platinum ring electrodes connected to a Grass S6 stimulator at 20 Hz (1 ms, 40 V).

Pretreatment with intravenous adrenaline or 6-hydroxydopamine

(−)-Adrenaline (400 μg/kg) was infused by means of a pump over a 40 min period into a brachial vein of chickens anaesthetized with sodium hexobarbitone. During this time anaesthesia was maintained as necessary with ether. The dissection, collection of blood, and removal of organs were performed as already described. Four chickens were pretreated with 6-hydroxydopamine hydrochloride 2 x 20 mg/kg given intravenously 14 days before and 2 x 50 mg/kg 7 days before the experiment; 100 mg was dissolved in 5 ml 0.001 N HCl which was gassed with nitrogen (schedule according to Thoenen & Tranzer, 1968).

Estimation of catecholamine concentrations of organs, plasma and perfusates

Organs were homogenized and extracted in 5% trichloroacetic acid (TCA). Heparinized blood was centrifuged at 0° C for 5 min and the plasma mixed with an equal volume of 10% TCA. After 20 min of extraction the mixture was centrifuged for 5 min and the catecholamines were estimated in the supernatant. The perfusates of the isolated hearts were immediately acidified with 1 N H_2SO_4 to achieve a pH of 3-4.

The catecholamines of tissues, plasma and perfusate were estimated fluorimetrically by the trihydroxindole method after adsorption on, and elution from, aluminium oxide as described previously (Lindmar & Muscholl, 1964). That the amines detected in tissues, plasma and perfusates by this method were, indeed, noradrenaline and adrenaline was established by paper chromatography according to the method described by Muscholl (1959).

Recoveries from a mixture of 200 ng noradrenaline and 50 ng adrenaline were 68 ± 5% for noradrenaline and 67 ± 10% for adrenaline ($n = 9$) when the mixture was added to the TCA-extract of the chicken heart. Similar recoveries (67 ± 5% for noradrenaline and 62 ± 5% for adrenaline) were obtained when the same mixture (200/50) was added to perfusate samples. It is concluded that under the present conditions any underestimation of the relatively small amounts of adrenaline was not significant. These recovery experiments are particularly important for the evaluation of the present results since it is known that the differential estimation of adrenaline and noradrenaline is difficult when the concentration of one amine exceeds that of the other (Häggendal, 1966; Callingham & Sharman, 1970). The catecholamine concentrations given in this study have not been corrected for their recoveries.

Experiments on isolated atria and on arterial blood pressure

The right atria were isolated and suspended in 60 ml of Tyrode solution bubbled with 95% O_2 and 5% CO_2 at 37° C. The spontaneous contractions were recorded with a force-displacement transducer on a Hellige Helcoscriptor. After 30 min of equilibration, noradrenaline or adrenaline was added every 3 min so that the final concentrations increased cumulatively from 5×10^{-9} to 5×10^{-5} M.

For measurement of arterial blood pressure chickens were anaesthetized with sodium pentobarbitone (200 mg/kg, i.m.). The body temperature was kept constant at 39.5-40.5° C by means of an electric pillow. The ischiadic artery and ischiadic vein were cannulated according to the method of Natoff & Lockett (1957). The arterial pressure was recorded on a Hellige compensation linear scriptor. Noradrenaline or adrenaline dissolved in saline was injected into the vein in volumes ranging from 0.65 to 1.1 ml.

Statistical analyses

Results are expressed as mean ± s.e. mean. The significance of the data was evaluated with Student's *t*-test. Regression equations and correlation coefficients (*r*) were calculated by the method of least squares using a computer programme. The number of experiments is *n*.

Results

Noradrenaline and adrenaline content of heart, spleen, brain, adrenal glands and blood plasma

Substantial amounts of both noradrenaline and adrenaline were found in all organs studied (Table 1). The adrenaline concentration expressed as percentage of noradrenaline plus adrenaline was highest in the adrenals (59%) and lowest in the spleen (16%). The relative adrenaline concentration of each organ was variable. However, there was no positive correlation when the concentrations in the hearts were compared with those of the spleens or brains from the same animal ($r < 0.36$). In 12 perfused hearts the mean noradrenaline concentration of the atria was 2.9 ± 0.4 fold higher than that of the ventricles. Essentially the same concentration ratio (2.6 ± 0.4 fold) was found for adrenaline (Table 1).

A total volume of approximately 30 ml blood could be obtained during exsanguination from a cannula located in the left ventricle. The mean concentrations of noradrenaline and adrenaline (Table 1) found when the total volume was removed in one step was 20 and 74 ng/ml, respectively, i.e. the adrenaline concentration was approximately four times higher than that of noradrenaline. In 14 animals the blood was collected in three consecutive 10 ml fractions. As shown in Table 2, the absolute concentrations of both amines increased considerably during exsanguination but the relative concentration of adrenaline remained constant. The concentration of adrenaline in the plasma was always significantly ($P < 0.001$) higher than it was in the adrenal glands.

Pretreatment of chickens with 6-hydroxy-

Table 1 The noradrenaline and adrenaline concentrations in various tissues of the adult chicken

Organ	n	*Organ weight (g) or plasma volume (ml)*	*Noradrenaline § (NA)*	*Adrenaline (Ad)*	*Adrenaline (% of NA plus Ad)*
Heart*	21	4.2 ± 0.2	175 ± 19	83 ± 11 ng/g	33.1 ± 3.3
Spleen	67	2.2 ± 0.1	477 ± 27	90 ± 8 ng/g	16.4 ± 1.3
Brain	7	1.0 ± 0.02	588 ± 49	236 ± 70 ng/g	26.3 ± 5.1
Adrenal glands	28	0.08 ± 0.01	2.7 ± 0.2	4.0 ± 0.3 mg/g	58.9 ± 2.7
Plasma†	39	15.4 ± 1.0	20 ± 4	74 ± 11 ng/ml	79.5 ± 2.0
Whole heart‡	12	5.5 ± 0.3	177 ± 31	50 ± 11 ng/g	23.0 ± 4.2
Atria	12	0.5 ± 0.04	366 ± 36	102 ± 23 ng/g	21.3 ± 4.1
Ventricles	12	5.0 ± 0.3	158 ± 31	45 ± 10 ng/g	23.7 ± 4.3

* Non-perfused hearts. † Plasma was obtained by exsanguination through a cannula inserted into the left ventricle.
‡ Perfused hearts. § Units of concentrations as in the following column.

dopamine caused a decrease of both noradrenaline and adrenaline in the heart and the spleen. In the heart the noradrenaline concentration declined from 175 ± 19 ng/g ($n = 21$) to 35 ± 3 ng/g ($n = 4$) (20%) and the adrenaline concentration from 83 ± 11 ng/g to 19 ± 8 ng/g (23%). Similar changes were obtained in the spleen, where the concentrations were reduced to 78 ± 23 ng/g (16%) and to 26 ± 7 ng/g (29%) of the control values (Table 1), respectively.

Output of noradrenaline and adrenaline evoked from isolated hearts by sympathetic nerve stimulation, tyramine or 'high K$^+$-solution'

Hearts isolated from anaesthetized chickens were perfused at 34°C with a constant volume of 24-25 ml/minute. The rate of contraction was 129 ± 4 beats/min ($n = 31$). During a 30 min period the resting output of noradrenaline was 0.018 ± 0.009 ng g^{-1} min^{-1} while that of adrenaline was –0.004 ± 0.002 ng g^{-1} min^{-1} ($n = 6$). All estimations of the resting output were carried out *prior* to sympathetic nerve stimulation or to infusions of tyramine or of high K$^+$-solution.

Stimulation of the right sympathetic nerves of isolated chicken hearts at 20 Hz for 1 min (1 ms, 40 V) caused a mean maximum increase of 119 beats/minute. The perfusate was collected during sympathetic nerve stimulation and for 1.5 min thereafter. Ten min after the first stimulation period, the procedure was repeated and again the perfusate was collected for a total of 2.5 minutes. Both stimulation perfusates were pooled to give a total volume of 125 ml It was found that sympathetic nerve stimulation evoked the output of 27 ± 5 ng/g noradrenaline (range 18 to 14) which represents about 15% of the total noradrenaline content of the heart. Moreover, sympathetic nerve stimulation caused an output of 7 ± 2 ng/g adrenaline (range 1.3 to 14.3) which represents 20 ± 5% of the sum of noradrenaline plus adrenaline and about 14% of the total adrenaline content of the heart (Table 3).

When tyramine (2.9 x 10^{-5} M) was infused for 30 min, the mean heart rate increased maximally from 111 to 210 beats/minute. The maximum increase was reached after 3-5 min of infusion and was followed by a gradual decline. The rate was still increased by 19 beats/min at the end of the tyramine infusion. During the 30 min exposure, tyramine caused a mean output of 48 ± 9 ng/g noradrenaline (range 18.6 to 145.6) and of 15 ± 2 ng/g adrenaline (range 2.6 to 39.9) which

Table 2 The noradrenaline and adrenaline concentration in the plasma during exsanguination

Plasma fractions	*Noradrenaline (NA) (ng/ml)*	*Adrenaline (Ad) (ng/ml)*	*Adrenaline (% of NA plus Ad)*
I + II + III	32 ± 8	100 ± 24	74 ± 2
I	16 ± 5	51 ± 14	77 ± 3
II	25 ± 6	99 ± 24	76 ± 3
III	51 ± 14	143 ± 37	71 ± 3

Chickens were exsanguinated by puncturing the left ventricle to remove 30-40 ml of blood, which was collected consecutively in 10 ml fractions I, II, and III. $n = 14$.

Table 3 The output of noradrenaline and adrenaline from the isolated heart of chicken in response to various stimuli and the remaining amine concentrations in the hearts

		Output of noradrenaline (NA) and adrenaline (Ad) into perfusate			*Concentration of NA and Ad remaining in the hearts†*		
*Stimulant**	n	*NA (ng/g)*	*Ad (ng/g)*	*Ad (% of NA plus Ad)*	*NA (ng/g)*	*Ad (ng/g)*	*Ad (% of NA plus Ad)*
Symp. nerve stim.	6	27 ± 5	7 ± 2	20 ± 5	157 ± 26	42 ± 10	22 ± 6
High K$^+$-solution	3	22 ± 2	9 ± 3	28 ± 8	123 ± 6	36 ± 16	22 ± 9
Tyramine	13	48 ± 9	15 ± 2	23 ± 4	160 ± 31	32 ± 5	19 ± 4

* The stimulants for noradrenaline and adrenaline output were sympathetic nerve stimulation (20 Hz, 2 x 1 min period), 'high K$^+$-solution' (108 mM K$^+$, 44 mM Na$^+$, 5 min infusion period) and tyramine (2.9 x 10^{-5} M, 30 min infusion period). ‡ The amine concentration was measured after the stimulation.

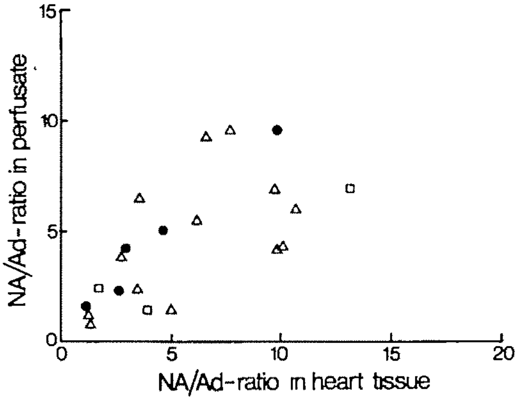

Figure 1 Isolated perfused heart of chicken. Variation of noradrenaline adrenaline ratio (NA/Ad) in stimulation perfusate with NA/Ad ratio in heart tissue. Output of NA and Ad was in response to sympathetic nerve stimulation (20 Hz, 1 ms, 40 V; (●)), tyramine (2.9×10^{-5} M; (△)) or 'high K^+-solution' (K^+ 108, Na^+ 44 mM, (□)). Ordinate scale, output of NA and Ad into perfusate expressed as NA/Ad ratio. Abscissa scale, Na and Ad content remaining in the hearts after the end of perfusion expressed as NA/Ad ratio.

represents 23 ± 4% of the sum of both amines (Table 3). The relative adrenaline concentration remaining in these hearts was 19 ± 4% which is approximately the same as that of the perfusate.

In three hearts the K^+ and Na^+ concentrations of the Tyrode solution were changed from 2.7 to 108 mM $[K^+]_0$ and from 149.3 to 44 mM $[Na^+]_0$ for 5 minutes. This 'high K^+-solution' caused immediate arrest of the hearts and liberated noradrenaline and adrenaline in all experiments. The proportion of adrenaline released was not significantly different from that remaining in the hearts (Table 3).

A correlation between the individual rates of noradrenaline output and those of adrenaline output was not detected. However a plot of the noradrenaline: adrenaline ratios in perfusates of stimulated hearts against the ratios found in the corresponding heart tissue (Figure 1) revealed a positive correlation with a regression coefficient of 0.55 (correlation coefficient 0.78).

The effect of in-vivo *pretreatment with (−)-adrenaline on the amine content in heart, spleen, plasma, and cardiac perfusate*

(−)-Adrenaline (10 μg $kg^{-1}min^{-1}$) was infused into a brachial vein for 40 minutes. The spleen and the first 10 ml fraction of blood were removed for immediate estimation of noradrenaline and adrenaline, whereas the amines of the heart were measured after perfusion for 30 min with tyramine (2.9×10^{-5} M).

After infusion of adrenaline (Figure 2) the content of this amine was greatly elevated in heart

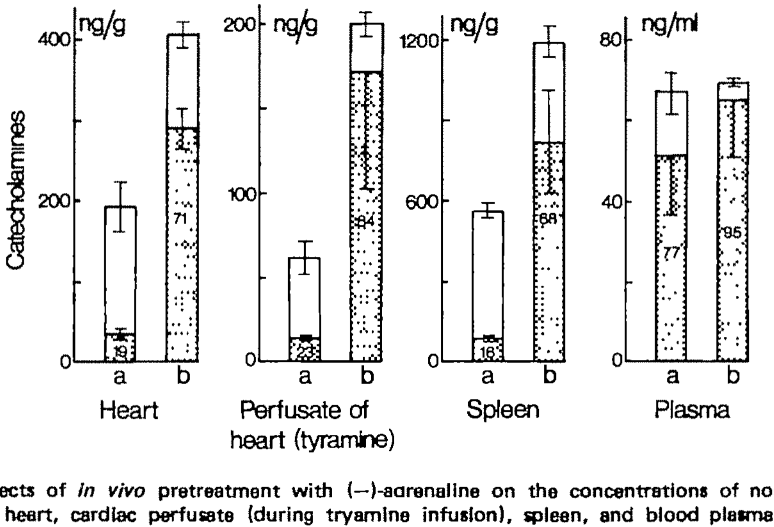

Figure 2 Effects of *in vivo* pretreatment with (−)-aarenaline on the concentrations of noradrenaline and adrenaline in heart, cardiac perfusate (during tryamine infusion), spleen, and blood plasma. Anaesthetized chickens received an infusion of 10 μg $kg^{-1}min^{-1}$ (−)-adrenaline for 40 minutes. Ordinate scale, noradrenaline (open bars) and adrenaline (stippled bars) concentrations in heart (after tyramine infusion), cardiac perfusate (during infusion of 2.9×10^{-5} M tyramine for 30 min), spleen, and blood plasma (1st 10 ml fraction during exsanguination, see methods sections and Table 2). Column (a) of each pair represent control values (no adrenaline pretreatment) and column (b) the values obtained after (−)-adrenaline pretreatment. The numbers within the bars indicate the % adrenaline concentration of the sum of both amines. The numbers of experiments for each column were 13, 3, 13, 3, 67, 3, 14, 3, (from left to right).

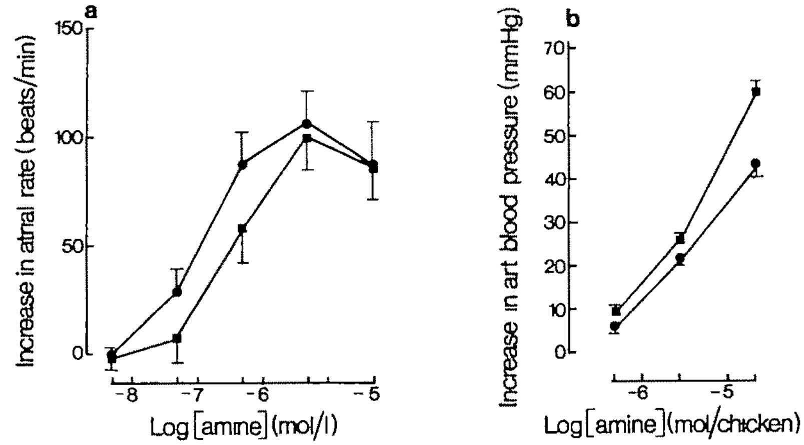

Figure 3 Comparison between cardiovascular potency of noradrenaline and adrenaline. (a) Cumulative concentration-response curves for noradrenaline (■) and adrenaline (●). Ordinate scale, increase in rate of contractions per min after exposure to various amine concentrations between 5×10^{-9} M and 5×10^{-5} M. Abscissa scale, molar concentrations of noradrenaline and adrenaline. (b) Dose-response curves for noradrenaline (■) and adrenaline (●). Ordinate scale, increase in arterial blood pressure (mmHg) after intravenous application of 0.5, 2.5 or 12.5 μmol per chicken. The weights of the chickens (1.0-1,2 kg) were almost the same. Each curve represents mean results from 6 atria or 6 anaesthetized chickens, respectively Vertical bars indicate s.e. mean. For further details see methods and results sections.

and spleen, whereas the noradrenaline content decreased slightly. In the plasma significant changes in the concentration ratio of the two amines were not detected. Thus adrenaline now predominated over noradrenaline not only in the plasma, but also in the heart and the spleen. The relative concentration of adrenaline in the heart (71%) was nearly the same (84%) as that found in the heart perfusate during tyramine infusion (Figure 2).

The sum of the noradrenaline and adrenaline concentrations was doubled in the heart and the spleen after pretreatment with (—)-adrenaline, whereas the total output of both amines released by tyramine into the cardiac perfusate was increased 3.5-fold. As in the experiments without adrenaline pretreatment, a significant spontaneous release from the perfused heart could not be detected.

Cardio-vascular potency of noradrenaline compared with that of adrenaline in the chicken

The spontaneous contractions of right atria isolated from 12 chickens were recorded isometrically. The atria were suspended in 60 ml Tyrode solution at 35°C. After equilibration for 30 min, noradrenaline (6 atria) or adrenaline (6 atria) was added to the solution every 3 min so that cumulative concentration-response curves were obtained. As shown in Figure 3, both amines caused a dose-dependent increase in rate of contraction. The ED_{50} of noradrenaline (6×10^{-7} M) and the slope of the curve were identical with those of adrenaline.

The effects of noradrenaline and adrenaline on the isometric tension development were studied on electrically driven (3 Hz) left atria. It was found that the amplitude of contractions was increased by noradrenaline (ED_{50} 4.5×10^{-7} M) and by adrenaline (ED_{50} 6×10^{-7} M) to the same extent. Intravenous injections of either noradrenaline or adrenaline in increasing doses elevated the arterial blood pressure of chickens anaesthetized with sodium hexobarbitone. As shown in Figure 3, both amines were about equally effective in raising the arterial blood pressure.

Discussion

In the present study it has been found that adrenaline as well as noradrenaline is the sympathetic transmitter in the chicken. The evidence was as follows: 1. Sympathetic nerve stimulation, 'high K^+-solution' and tyramine caused the release of both amines from isolated perfused hearts (Table 3). 2. Chemical sympathectomy by pretreatment with 6-hydroxydopamine reduced the concentrations of noradrenaline and adrenaline in heart and spleen. Degeneration of adrenergic neurones in the chicken heart following treatment with 6-hydroxydopamine was demonstrated by Bennett, Malmfors & Cobb (1973). 3. Both noradrenaline and adrenaline concentrations in the atria were two to three times higher than those in the ventricles. This corresponds with the relatively high overall density of the atrial adrenergic nerve plexus when compared with the ventricular innervation of the chicken heart (Bennett & Malmfors, 1970). 4. The increases of the arterial blood pressure and the stimulating effects on isolated atria evoked by noradrenaline and adrenaline showed nearly identical cardio-vascular potencies for both amines (Figure 3). This confirms the recent observation that the noradrenaline sensitivity of chicken left atria was not significantly different from the adrenaline sensitivity (Bennett & Malmfors, 1974). To our knowledge, a functional role for two transmitters in the peripheral sympathetic nervous system has not been described for any other species. In the frog the transmitter in the peripheral sympathetic nervous system is adrenaline, whereas in the mammal it is noradrenaline, but in neither of these classes do both transmitters function together as they appear to do in the chicken. Although relatively small amounts of adrenaline have been found in mammalian organs (see below), only noradrenaline was released from them. This holds true even when, for example, a maximally effective stimulant, such as dimethylphenyl-piperazinium iodide (DMPP) is applied to the rabbit isolated heart (Lindmar, Löffelholz & Muscholl, 1968). If, in this latter study, a release of adrenaline had been missed because of the limitations of estimation, it could have been only 1% of that of noradrenaline or less. There are striking differences between mammalian and frog hearts on the one hand (Holzbauer & Sharman, 1972) and chicken hearts on the other. Firstly, in the chicken heart both amines are present in relatively high concentrations so that there is twice as much noradrenaline as adrenaline. The predominance of noradrenaline over adrenaline has been previously reported (Ignarro & Shideman, 1968; Sturkie & Poorvin, 1973). Secondly, the sum of the absolute noradrenaline and adrenaline concentrations in the chicken heart is much lower than that of either mammalian and frog hearts.

The small adrenaline concentrations that have been detected in mammalian organs (Holzbauer & Sharman, 1972) were shown to originate from the circulation (Strömblad, 1961; Strömblad & Nickerson, 1961; Coleman & Glaviano, 1963: Muscholl, Kiefer & Lindmar, 1969). On the other hand, the phenylethanol-*N*-methyltransferase activity (PNMT) in the chicken heart was much higher than that in the rabbit heart (Lindmar & Wolf, 1975). Furthermore, Lindmar *et al.* (unpublished observations) have obtained some indirect evidence for a neuronal localization of this enzyme. It is, nevertheless, likely that part of the adrenaline stored in heart and spleen of the chicken has been removed from the circulation since the plasma levels of adrenaline were remarkably high (Table 2) and the nerves were able to concentrate (−)-adrenaline from the circulation (Figure 2). Moreover, the newly incorporated (−)-adrenaline was preferentially released by tyramine. On the other hand the possibility cannot be excluded that some of the catecholamines found in sympathetically innervated organs are located in non-neuronal catecholamine-storing structures which have been described in embryonic (Enemar, Falck & Håkanson, 1965) and mature chicken hearts (Bennett & Malmfors, 1970). However, the latter authors have pointed out that the number of round fluorescent cells in the heart of the adult chicken is relatively low when compared to mammalian hearts.

The nearly four-fold predominance of adrenaline over noradrenaline in the plasma was maintained throughout the gradual exsanguination although the noradrenaline and adrenaline concentrations in the plasma rose markedly, presumably because of an acute secretion of medullary hormones induced by hypovolemia. Lin & Sturkie (1968) also found about four times more adrenaline than noradrenaline in the plasma but the absolute amine concentrations were more than 10 times lower than those found in the present study. Although physiological plasma levels are unknown, it can be assumed that the values obtained at the beginning of exsanguination (adrenaline 51 ng/ml, noradrenaline 16 ng/ml, Table 2) were close to physiological amine concentrations. Thus, the chicken has a much higher plasma catecholamine concentration than mammals where the concentrations of noradrenaline and adrenaline are below 1 ng/ml (Holzbauer & Sharman, 1972). The high plasma levels of catecholamines suggest that, in the chicken, the medullary hormones play an important role in regulating cardiac performance and blood pressure as was previously assumed (Karg & Schams, 1966).

We wish to thank Miss U. Wolf for her excellent technical assistance. Part of this work was the Student Research Project of one of the authors (V.P.D.) and was supported by the University of Connecticut, Health Center, Farmington, Conn. U.S.A.

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(Received April 24, 1975.
Revised May 27, 1975.)

Br. J. Pharmac. (1975), 55, 351-358

BEHAVIOURAL CHANGES INDUCED IN CONSCIOUS MICE BY INTRACEREBROVENTRICULAR INJECTION OF CATECHOLAMINES, ACETYLCHOLINE AND 5-HYDROXYTRYPTAMINE

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1 A simple method of injecting soluble substances into the lateral ventricle of the brain of the conscious mouse is described.

2 The effect of various doses of noradrenaline, dopamine, acetylcholine, 5-hydroxytryptamine given into the right lateral brain ventricle were tested on locomotor and exploratory activities of mice.

3 Noradrenaline in a dose of 0.1 μg increased locomotor activity. This effect was prevented by phenoxybenzamine but not by propranolol.

4 Higher doses of noradrenaline (1 or 10 μg) decreased locomotor and exploratory activities. Propranolol but not phenoxybenzamine abolished these effects.

5 Dopamine (0.1 or 1 μg) increased locomotor activity. The higher doses also induced tremor.

6 The highest dose of dopamine tested (10 μg) elicited stereotypical behaviour.

7 All the behavioural phenomena induced by 0.1 μg and 10 μg of dopamine were blocked by pimozide.

8 Acetylcholine (1 and 10 μg) and 5-hydroxytryptamine (1 μg) inhibited locomotor and exploratory activity.

9 The effects of 1 and 10 μg of acetylcholine were abolished by atropine (5 mg/kg i.p.). Methysergide (5 mg/kg i.p.) had no influence on the effects of 5-hydroxytryptamine (1 μg).

Introduction

In the last 25 years several authors have studied the behavioural effects of biogenic amines. These amines have been injected intraventrically, intracisternally, and into various brain structures in order to circumvent the blood-brain barrier. Adrenaline, noradrenaline (NA) and dopamine have been shown to depress a variety of different types of behaviour in dogs, cats, chickens, sheep, monkeys and rats (Leimdorfer, 1950; Feldberg & Sherwood, 1954; Palmer, 1959; Schain, 1961; Wada, 1962; Traczyk, 1964; Marley & Stephenson, 1969, 1970; Grunden & Marley, 1970). Amongst the changes observed by these authors were decreased locomotor activity, stupor, sleepiness, anaesthesia, catalepsy and sleep.

Other authors have described behavioural activation in rats after intraventricular administration of NA or dopamine (Segal & Mandell, 1970; Malec & Kleinrok, 1972; Stern & Zwick, 1973) or after intrahypothalamic or intrastriatal NA or dopamine injection (Benkert & Köhler, 1972). In this laboratory it has been shown that lower doses of NA applied intraventricularly cause increased locomotor activity in rats, but that higher doses elicit a stuporous syndrome. It has also been shown that the excitatory effects of NA in rats are dependent upon normal genetically determined exploratory activity (Herman, 1970). 5-Hydroxytryptamine (5-HT) and acetylcholine (ACh) decreased locomotor activity in rats, when injected intraventricularly (Herman, Kmieciak-Kołada, Słomińska-Żurek & Szkilnik, 1972; Herman, 1973).

The present experiments attempted to answer two questions: are the behavioural effects of NA, dopamine, ACh and 5-HT in mice similar to those described in rats; and are these effects pharmacologically specific?

Methods

The experiments were carried out on male, white, Swiss mice, weighing 20 g, 1 month old supplied by the Central Animal Farm of the Silesian School of Medicine.

Drugs

Noradrenaline bitartrate (Sigma), dopamine hydrochloride (Sigma), acetylcholine iodide (Chemapol), and 5-hydroxytryptamine (serotonin creatinine sulphate, Koch-Light) were injected intracerebroventricularly (i.c.v.). Pimozide (Janssen) was administered intraperitoneally. Doses of these substances are expressed as free bases.

Phenoxybenzamine hydrochloride (Smith Kline & French), propranolol hydrochloride (ICI), methysergide dimaleate (Deseril, Sandoz) and atropine sulphate (Polfa) were injected intraperitoneally. Doses of these substances were dissolved in 0.9% w/v NaCl solution (saline), except for methysergide, which was obtained from ampoules of Deseril.

Intraventricular injection technique

Mice were prepared as follows. Under light ether anaesthesia an incision was made along the mid-line of the skull. The bones were cleaned of connective tissue and the superior and transverse venous sinuses were identified. A point 3 mm from the confluence of the two sinuses and from the sagittal sinus of the right side was found. Here a small hole in the skull was made with an intramuscular needle with a sharp end. The hole was made by rotary movements of the needle. Twenty-four hours after this operation an intraventricular injection was performed using a 25 gauge needle with a sharp (17°) point, attached to a 1 µl Hamilton microsyringe 7001 N fixed to a stand, which allows vertical movements of the syringe. The plastic tube covered the whole needle except for the terminal 3 mm. The needle was left in place for approximately 10 s after injection. The procedure was checked by injection of 1 µl of methylene blue solution into the right lateral ventricle of the brain in 100 mice each weighing 20 grams. The place of puncture was examined and the presence of dye in the lateral ventricles was looked for by means of a hand lens after the animal had been killed and the brain sectioned. Of the injections, 8% were found not to have entered the ventricle. In these animals ataxic movements and head turning were observed. In the course of experiments if these behavioural changes suggested a faulty injection, the point of puncture and the walls of the lateral ventricle were examined macroscopically. If damage was observed in the tissues around the ventricle, the results obtained in these animals were discarded.

Observation of behaviour

Each mouse was placed in a glass cage, dimensions 40 x 25 x 25 cm, for 10 minutes. During this period the amount of time spent walking, washing, sniffing and in immobility was measured in seconds with a stop-watch to the nearest 1 second. These types of behaviour were classified as locomotor activity. The duration of tremors and stereotyped movements of the head were measured when these phenomena appeared. The number of rearings was also counted, rearing was considered as an exploratory activity.

Experimental design

Biogenic amines were dissolved in freshly prepared saline (0.9% w/v NaCl solution) a few seconds before the intraventricular injection. One mouse was injected with one dose of biogenic amine in the volume of 1 µl, the second animal with 1 µl of saline. The behaviour of both mice was measured immediately after intracerebroventricular injection. In a second series of experiments antagonists of biogenic amines were injected intraperitoneally in saline in a volume of 1 ml/kg. One hour later the behaviour of the animals was observed either after the antagonist alone or after intraventricular injection of biogenic amine. In other experiments the behavioural phenomena were measured in intact animals and compared with those seen in intraventricularly sham-injected mice and in animals injected intraventricularly with saline. Finally some groups of animals were injected intraventricularly with NA and immediately afterwards with dopamine. All mice were used only once. All experiments were performed between 09 h 00 min and 12 h 00 min during a period of 1.5 years. The statistical significances of the changes observed were assessed by Student's *t*-test.

Results

Doses of substances blocking the pharmacological effects of biogenic amines used in these experiments did not affect the components of behaviour of mice assessed. Pimozide in a dose of 0.1 mg/kg was an exception and was not used in this dose in further experiments (Table 1).

NA (0.1 µg i.c.v.) increased walking time and decreased immobility. These effects were abolished by phenoxybenzamine (5 mg/kg i.p.) but not by propranolol (10 mg/kg i.p.). A higher dose of NA (1 μg) decreased walking and cleaning and abolished the sniffing and rearings, and increased immobility. Phenoxybenzamine did not change these effects. Propranolol reversed the behavioural effects of this dose of NA. The highest dose of NA (10 μg) caused complete immobility of mice. Phenoxybenzamine did not affect this phenomenon. Propranolol inhibited it (Table 2).

Dopamine (0.1 μg) increased the time spent walking and cleaning and the number of rearings and decreased the time spent immobile. These effects of dopamine were inhibited by pimozide. Dopamine in a dose of 1 μg increased the time of walking, decreased the time of immobility and induced tremors. Pimozide blocked these phenomena. After a dose of 10 μg of dopamine stereotyped movements of the head were observed. This type of behaviour was blocked by pimozide (Table 3).

Sham intraventricular injection caused a significant decrease in walking time. Intracerebroventricular injection of saline caused a decrease in walking time, in the number of rearings, and an increase in time spent immobile (Table 4). Simultaneous injections of NA and dopamine (0.1 μg) increased walking time and sniffing, and decreased immobility. The higher dose (1 μg) of both catecholamines administered i.c.v. simultaneously caused a decrease of walking time and of the number of rearings, suppressed cleaning and increased the time spent in sniffing and immobility (Table 4).

ACh in a dose of 0.1 μg increased the time spent sniffing and in a dose of 1 μg decreased walking time and increased the time spent immobile. The same effects, together with a decrease in time spent sniffing and suppression of rearing, were observed after a dose of 10 μg of ACh. These effects of ACh were abolished by atropine (Table 5).

5-HT in a dose of 0.1 μg caused no changes in behaviour but in a dose of 1 μg decreased walking time, suppressed cleaning and rearing, and increased the time spent immobile. Methysergide had no influence on these effects of 5-HT (Table 6).

Discussion

The results presented show that sham intracerebroventricular injection or injection of saline to conscious mice depress both locomotor and exploratory activity. Therefore in interpreting the behavioural effects of intraventricularly injected substances the fact that the results are

Table 1 Behaviour of mice after injections of drugs blocking pharmacological effects of biogenic amines

Control	Time (s) Walking	Time (s) Cleaning	Time (s) Sniffing	Time (s) Immobility	Number of rearings	Drug (mg/kg i.p.)	Time (s) Walking	Time (s) Cleaning	Time (s) Sniffing	Time (s) Immobility	Number of rearings
Saline 0.9% (1 ml/kg of body weight i.p.)	200 ± 13.4	30 ± 7.1	25 ± 5.0	345 ± 15.5	10 ± 1.8	Phenoxy-benzamine (5)	210 ± 15.6 $P > 0.35$	35 ± 10.1 >0.35	25 ± 5.1 >0.4	320 ± 15.4 >0.05	7 ± 1.2 >0.15
	230 ± 34.2	60 ± 15.3	35 ± 6.7	275 ± 28.3	16 ± 4.4	Propranolol (10)	238 ± 22.5 $P > 0.4$	62 ± 12.5 >0.45	40 ± 7.1 >0.3	260 ± 27.7 >0.4	16 ± 2.7 >0.35
	230 ± 19.0	45 ± 7.4	20 ± 4.2	305 ± 19.3	21 ± 2.0	Pimozide (0.05)	240 ± 26.7 $P > 0.3$	30 ± 8.0 >0.1	30 ± 6.8 >0.1	300 ± 27.7 >0.4	24 ± 3.7 >0.25
	290 ± 21.1	40 ± 10.4	20 ± 3.0	250 ± 21.6	32 ± 2.1	Pimozide (0.1)	230 ± 24.7 $P > 0.05$	20 ± 6.3 >0.05	10 ± 2.5 <0.025	340 ± 24.6 <0.0125	28 ± 2.9 >0.15
	210 ± 33.7	60 ± 11.1	25 ± 3.0	315 ± 45.7	13 ± 1.7	Methysergide (5)	240 ± 40.2 $P > 0.25$	50 ± 8.1 >0.5	30 ± 4.2 >0.15	280 ± 41.2 >0.25	20 ± 4.4 >0.05
	380 ± 13.8	20 ± 4.8	10 ± 3.3	190 ± 10.7	32 ± 5.8	Atropine (5)	340 ± 25.8 $P > 0.1$	30 ± 15.7 >0.25	10 ± 3.3 >0.5	220 ± 21.0 >0.15	40 ± 5.8 >0.2

$n = 10$ in each group. Results expressed as a mean ± s.e.

Table 2 Behavioural effects of noradrenaline (NA) in mice

	Group											
Component of behaviour	*I* *Saline (control)*	*II* *NA (0.1 μg)*	*III* *Pb (5 mg/kg) plus NA (0.1 μg)*	*IV* *Prop (10 mg/kg) plus NA (0.1 μg)*	*V* *Control*	*VI* *NA (1 μg)*	*VII* *Pb (5 mg/kg) plus NA (1 μg)*	*VIII* *Prop (10 mg/kg) plus NA (1 μg)*	*IX* *Control*	*X* *NA (10 μg)*	*XI* *Pb (5 mg/kg) plus NA (10 μg)*	*XII* *Prop (10 mg/kg) plus NA (10 μg)*
Walking (s)	45 ± 12.4	95 ± 11.8 *P* I/II <0.01	50 ± 10.0 *P* II/III <0.01	100 ± 9.7 *P* II/IV >0.5	153 ± 32	59 ± 12 *P* V/VI <0.025	50 ± 12 *P* VI/VII >0.5	110 ± 10 *P* VI/VIII <0.01	141 ± 23	0	0	110 ± 16
Cleaning (s)	10 ± 4.4	25 ± 8.3 >0.05	0	15 ± 4 >0.2	53 ± 23	15 ± 3 <0.025	10 ± 33 >0.3	40 ± 6 <0.005	45 ± 20	0	0	20 ± 6
Sniffing (s)	5 ± 2.6	15 ± 4.7 >0.05	10 ± 5 >0 2	5 ± 2 >0.05	20 ± 9	0	10 ± 4	10 ± 3	48 ± 17	0	0	0
Immobility (s)	540 ± 10.3	465 ± 17.2 <0.0005	540 ± 21 <0.01	480 ± 8 >0.5	374 ± 60	526 ± 16 <0.025	530 ± 12 >0.5	440 ± 9 <0.0005	366 ± 40	600 ± 0 *P* IX/X <0.0005	600 ± 0	470 ± 15 *P* X/XII <0.0005
Number of rearings	2 ± 0.6	4 ± 1.0 >0.1	0	3 ± 1 >0.1	2 ± 1	0	2 ± 1	3 ± 1	2 ± 0	0	0	2 ± 1

$n = 10$, except groups I, II where $n = 20$. Results expressed as a mean ± s.e.
Pb = phenoxybenzamine; Prop = propranolol.

Table 3 Behavioural effects of dopamine (DA) in mice

					Group				
Component of behaviour	*I Saline control*	*II DA (0.1 μg)*	*III Pim (0.05 mg/kg) plus DA (0.1 μg)*	*IV Control*	*V DA (1 μg)*	*VI Pim plus DA (1 μg)*	*VII Control*	*VIII DA (10 μg)*	*IX Pim plus DA (10 μg)*
Walking (s)	42 ± 7	124 ± 14 *P* I/II <0.0005	70 ± 15 *P* II/III <0.01	70 ± 14	115 ± 20 *P* IV/V <0.05	84 ± 21 *P* V/VI >0 2	85 ± 30	120 ± 25 *P* VII/VIII >0.2	100 ± 25 *P* VIII/IX >0 3
Cleaning (s)	0	6 ± 3 <0.05	6 ± 3	10 ± 4	8 ± 3	45 ± 15 <0.02	15 ± 5	0	3 ± 2
Sniffing (s)	11 ± 5	16 ± 6 >0.3	16 ± 5	20 ± 5	36 ± 10	44 ± 11	20 ± 10	7 ± 4 >0.15	11 ± 5
Tremor (s)	0	0	0	0	161 ± 34	0	0	0	0
Immobility (s)	546 ± 6	453 ± 20 <0.0025	508 ± 15 <0.05	500 ± 10	280 ± 42 <0.0005	470 ± 20 <0.0002	480 ± 20	360 ± 34 <0.01	476 ± 28 <0.02
Stereotyped movements of the head	0	0	0	0	0	0	0	115 ± 28	0
Number of rearings	1 ± 0	3 ± 1	1 ± 0	1 ± 0	2 ± 0	0	2 ± 1	2 ± 1	2 ± 1

n = 10. Results expressed as a mean ± s e
Pim = pimozide.

Table 4 Behavioural effects of noradrenaline (NA) and dopamine (DA) injected simultaneously intraventricularly (i.c.v.) in mice

	Group							
Component of behaviour	*I* *Intact animals*	*II* *Sham I.c.v. injection*	*III* *Intact animals*	*IV* *Saline I.c.v.*	*V* *Saline I.c.v.*	*VI* *NA (0.1 μg) plus DA 0.1 μg*	*VII* *Saline I.c.v.*	*VIII* *NA (1 μg) plus DA (1 μg)*
Walking (s)	299 ± 54	153 ± 32 *P* I/II <0.025	259 ± 46	130 ± 25 *P* III/IV <0.025	90 ± 12	110 ± 25 *P* V/VI >0.2	170 ± 17	50 ± 12 *P* VII/VIII <0.0005
Cleaning (s)	36 ± 10	53 ± 23 >0.25	30 ± 7	40 ± 11 >0.2	36 ± 8	0	60 ± 12	0 <0.0005
Sniffing (s)	40 ± 17	20 ± 9 >0.2	50 ± 18	20 ± 15 >0.1	20 ± 4	165 ± 20 <0.0005	20 ± 12	55 ± 9 <0.005
Immobility (s)	225 ± 59	374 ± 60 >0.1	261 ± 50	410 ± 40 <0.005	464 ± 42	326 ± 28 <0.0025	350 ± 19	495 ± 11 <0.0005
Number of rearings	14 ± 7	2 ± 1 <0.05	11 ± 4	2 ± 1 <0.05	2 ± 1	4 ± 1 >0.2	7 ± 0.8	2 ± 0.7 <0.0025

n = 20 (group I.IV), *n* = 10 (group V-III). Results expressed as a mean ± s.e.

Table 5 Behavioural effects of acetylcholine (ACh) in mice

	Group							
Component of behaviour	*I* *Saline (control)*	*II* *ACh (0.1 μg)*	*III* *Control*	*IV* *ACh (1 μg)*	*V* *Atropine (5 mg/kg) plus ACh (1 μg)*	*VI* *Control*	*VII* *ACh (10 μg)*	*VIII* *Atropine plus ACh (10 μg)*
Walking (s)	80 ± 12	62 ± 4 *P* I/II >0.1	114 ± 22	52 ± 15 *P* III/IV <0.0125	70 ± 12 *P* IV/V >0.15	116 ± 31	47 ± 7 *P* VI/VII <0.05	96 ± 13 *P* VII/VIII <0.005
Cleaning (s)	6 ± 4	14 ± 6 >0.1	10 ± 6	7 ± 5 >0.35	5 ± 2 >0.3	10 ± 5	10 ± 5 >0.9	10 ± 5 >0.9
Sniffing (s)	36 ± 4	50 ± 2 <0.01	52 ± 30	55 ± 30 >0.45	50 ± 25 >0.9	52 ± 11	7 ± 3 <0.0025	45 ± 5 <0.0005
Immobility (s)	480 ± 4	474 ± 15 >0.4	420 ± 30	485 ± 16 <0 05	475 ± 20 >0.9	420 ± 35	530 ± 23 <0.0025	449 ± 40 >0.1
Number of rearings	2 ± 0	2 ± 0 >0.9	3 ± 1	2 ± 1 >0.5	2 ± 1 >0.9	2 ± 1	0	2 ± 1

n = 10. Results expressed as a mean ± s.e.

Table 6 Behavioural effects of 5-hydroxytryptamine (5-HT) in mice

			Group		
	I	II	III	IV	V
Component of behaviour	*Saline (control)*	*5-HT (0.1 μg)*	*Control*	*5-HT (1 μg)*	*Methysergide (5 mg/kg) plus 5-HT (1 μg)*
Walking (s)	102 ± 21	72 ± 16 *P* I/II >0.1	86 ± 8	8 ± 2 *P* III/IV <0.0005	10 ± 5 *P* IV/V >0.3
Cleaning (s)	5 ± 3	15 ± 6 >0.05	3 ± 1	0 <0.01	0
Sniffing (s)	35 ± 7	30 ± 7 >0 35	41 ± 7	30 ± 7 >0.1	0
Immobility (s)	458 ± 23	498 ± 21 >0 1	470 ± 6	560 ± 8 <0 0005	590 ± 7 <0 01
Number of rearings	1 ± 0	1 ± 0 >0.9	3 ± 1	0 <0.01	0

n = 10. Results expressed as mean ± s.e.

obtained in animals already depressed by the experimental procedure has to be taken into consideration.

The behavioural effects of NA depend upon the dose. Low doses of NA increase, and higher doses decrease, locomotor activity. The behavioural effects of NA observed here in mice are very similar to those described in rats (Herman, 1970). These two dose-dependent central effects of NA are suppressed or even reversed by different adrenoceptor blocking agents. Andén & Strömbom (1974) concluded that phenoxybenzamine, but not phentolamine or propranolol, can be used to block selectively central and peripheral NA receptors in behavioural experiments. Our results indicating that phenoxybenzamine blocks the increased locomotor activity induced by low doses of NA are in agreement with this view. But in our experiments propranolol reversed the inhibition of locomotor and exploratory activities elicited by 1 μg of NA and suppressed the inhibition caused by 10 μg of NA. These facts suggest that behavioural depression caused by an excess of exogenous NA may be influenced by β-adrenoceptor blockade.

Dopamine also affects the behaviour of mice in a dose-dependent manner. It causes increased locomotor and exploratory activity in the lowest dose (0.1 μg), and an increase of locomotion and the appearance of tremors in a medium dose (1 μg). The highest dose used (10 μg) induced stereotyped behaviour. This last phenomenon and the observation that the behavioural effects of dopamine were blocked by pimozide, a drug which is considered to be a specific blocker of dopamine receptors in the brain (Andén, Butcher, Corrodi, Fuxe & Ungerstedt, 1970), suggest that the behavioural effects of exogenous dopamine applied intraventricularly are specific to dopamine receptors. Central dopamine action in mice resembles its effects in rats (Malec & Kleinrok, 1972). Simultaneous intraventricular injection of NA and dopamine in equal doses showed no synergistic or antagonistic effects in terms of the behavioural changes observed after the application of a single amine.

ACh decreased locomotor and exploratory activity in mice in a similar manner to that previously described (Herman *et al.*, 1972). These effects may be of muscarinic nature since they were abolished by atropine.

5-HT elicited depressant behavioural effects similar to those described in rats (Herman, 1973). However, methysergide considered as a 5-HT antagonist (McKeon Jr., 1967, Boakes, Bradley, Briggs & Dray, 1970) did not inhibit the behavioural phenomena evoked by 5-HT.

In conclusion it may be said that behavioural effects of catecholamines, ACh and 5-HT administered intraventricularly are similar to those described in rats by several authors. These effects, with the exception of 5-HT, can be blocked by antagonists of the amine investigated.

The author expresses his gratitude for a generous supply of substances, to Janssen (pimozide), ICI (propranolol), Smith, Kline & French (phenyoxybenzamine), Polfa (atropine) and Sandoz (Deseril). This work was supported financially by the Polish Academy of Sciences.

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(Received April 25, 1975.)

Br J. Pharmac. (1975), 55, 359-368

ANTIARRHYTHMIC, HAEMODYNAMIC AND METABOLIC EFFECTS OF 3α-AMINO-5α-ANDROSTAN-2β-OL-17-ONE HYDROCHLORIDE IN GREYHOUNDS FOLLOWING ACUTE CORONARY ARTERY LIGATION

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1 The antiarrhythmic, haemodynamic and metabolic effects of a new amino steroid, ORG6001, have been investigated in experimental acute myocardial infarction in anaesthetized greyhounds.

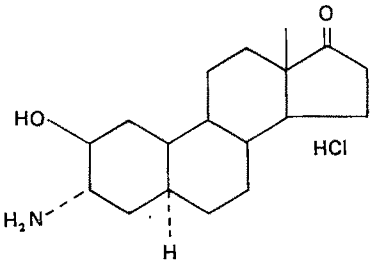
2 ORG6001 administered either intravenously (1-10 mg/kg) or orally (50 mg/kg) significantly reduced the incidence of ventricular ectopic beats in the first 30 min after ligation of the left anterior descending coronary artery.

3 In dogs pretreated with ORG6001, metabolic changes indicative of myocardial ischaemia (lactate production and potassium efflux) were less marked than those occurring in control animals.

4 Antiarrhythmic doses of ORG6001 caused only minimal transient haemodynamic effects.

5 These results suggest that ORG6001 may possess distinct advantages over presently-used antiarrhythmic drugs in the prevention and treatment of the early arrhythmias which occur after myocardial infarction.

Introduction



Formula of ORG6001
(3α-amino-5α-androstan-2β-ol-17-one HCl)

It has recently been reported that ORG6001 (3α-amino-5α-androstan-2β-ol-17-one hydrochloride) protects animals from cardiac arrhythmias induced either by aconitine, by ouabain or by multiple stage coronary artery ligation one day previously (Vargaftig, Sugrue, Buckett & Van Riezen, 1975). In addition, these workers have stated that ORG6001 may possess advantages over lignocaine since it has less adverse haemodynamic effects and is active orally. Acute one-stage ligation of the left anterior coronary artery in anaesthetized greyhounds results in reproducible and marked ventricular ectopic activity for 30 min after the occlusion and, in addition, causes metabolic changes in the venous blood draining the ischaemic region which do not occur in blood draining the remaining, essentially normal, areas of myocardium (Marshall, Parratt & Ledingham, 1974; Marshall & Parratt, 1974). The purpose of the present work was to investigate in this experimental model the haemodynamic and antiarrhythmic effects of ORG6001, administered both intravenously and orally, and to assess whether the drug was capable of modifying the metabolic consequences of acute coronary artery ligation.

Methods

Experiments were carried out using twenty-nine greyhounds of either sex weighing between 26 and 31 kg. Anaesthesia was induced by intravenous administration of sodium thiopentone

(20 mg/kg). After endotracheal intubation, respiration was applied from a positive-pressure ventilation pump (25 strokes/min), with 100% oxygen containing 0.5-1.0% trichlorethylene. The tidal volume of the pump was adjusted to maintain an arterial $P\text{CO}_2$ of 35-40 mmHg (1 mmHg = 1.33 mbar). Reflex movements were prevented by the intermittent intramuscular administration of suxamethonium chloride (100 mg).

Catheters were placed in the descending aorta (via the right femoral artery), the right atrium (via the right saphenous vein) and the coronary sinus (via the left external jugular vein). A catheter-tip transducer (Millar Instruments, Inc., Houston, Texas) was inserted into the lumen of the left ventricle (via the left carotid artery in the neck) for the measurement of left ventricular pressure and also d*P*/d*t* (using an Elema-Schonander differentiating circuit). The frequency response of this transducer system is flat to 200 Hz. Records of left ventricular pressure at high gain allowed accurate assessments to be made of left ventricular end-diastolic pressure (LVEDP). The correct location of all catheters was confirmed using a Siemen's image intensifier. Cardiac output was measured by dye-dilution, indocyanine green (2.5 mg) being injected as a bolus into the right atrium and blood withdrawn at a constant rate through a densitometer (Waters Co., Rochester, Minnesota). This blood was immediately replaced. All cardiac output determinations were made in duplicate.

The heart was exposed through a left thoracotomy and the pericardium overlying the anterolateral aspect of the heart was incised. Blood flow in the circumflex branch of the left coronary artery was measured with a Nycotron 372 electromagnetic flow meter using a calibrated probe of 1.5-2.5 mm diameter. The anterior descending branch of the left coronary artery, at a point distal to the septal artery branch, was prepared for ligation with minimum dissection. A major branch of the main vein adjacent to the artery (the anterior coronary vein) was catheterised using the Seldinger technique with a 10 cm Longdwel teflon catheter (size 20G). This local coronary vein catheter was not tied in position and was manipulated until its tip lay well into the region of the myocardium which was to be made ischaemic. It has been shown that such a coronary vein catheter, after coronary artery ligation, drains blood predominantly from the ischaemic region (Fisher, Heimbach, Ledingham, Marshall & Parratt, 1973; Marshall *et al.*, 1974).

Blood samples were taken, without exposure to air, at regular intervals and were analysed for oxygen and carbon dioxide tensions, oxygen content and pH, as outlined by Ledingham, McBride, Parratt & Vance (1970), except that the value used for haemoglobin oxygen-binding capacity was 1.39. Blood samples from the aorta, coronary sinus and coronary vein were taken immediately before and 30 min after coronary artery ligation and were analysed for lactate (Hohorst enzymatic method using a Boehringer test combination) and for plasma potassium using standard flame photometry. Potassium determinations were discarded if there was any obvious haemolysis.

After a stabilisation period, the ligature was tied in one stage and the number of ventricular ectopic beats counted during each 5 min period for 30 min; no blood samples were taken during this time since manipulation of the coronary venous catheter sometimes itself induced arrhythmias. No arrhythmias occurred in any of the animals after 30 minutes.

In order to investigate the possible anti-arrhythmic activity of ORG6001, the following protocol was used:

GROUP I–Intravenous ORG6001 (10 mg/kg), 15 min prior to ligation ($n = 6$)
GROUP II–Intravenous ORG6001 (10 mg/kg), 1.5 h prior to ligation ($n = 6$)
GROUP III–Intravenous ORG6001 (1.0 mg/kg), 15 min prior to ligation ($n = 5$)
GROUP IV–Oral ORG6001 (50 mg/kg) 4 h prior to ligation ($n = 7$)
GROUP V–Oral placebo (lactose), 4 h prior to ligation ($n = 5$)

Groups IV and V were studied double-blind and the key to the code was not revealed until all the results had been collated. One dog (which was revealed later to be in Group V) fibrillated during left ventricular catheterisation and was thus discounted from the trial. The results obtained in these groups of dogs were compared with those previously obtained in control and lignocaine-treated animals (Marshall & Parratt, 1974). At the end of each experiment, a bolus of diffusible dye was injected at a pressure of 50 mmHg into the peripheral stump of the ligated coronary artery. The dog was immediately fibrillated with potassium chloride and the dyed muscle quickly excised and weighed. The mass of this dyed ischaemic area of muscle was expressed as a percentage of the free ventricular wall.

Systemic arterial pressure (pulsatile and meaned by electronic integration), mean right atrial pressure, left ventricular pressure and d*P*/d*t*, LVEDP, left circumflex coronary blood flow and the electrocardiogram (standard limb lead II) were recorded on an Elema-Schonander ink-jet writing recorder (Mingograph 81).

Myocardial oxygen availability and consumption were calculated as outlined by Marshall &

Parratt (1973) and cardiac work, peripheral vascular resistance, whole body oxygen consumption and oxygen extraction as described by Ledingham, Parratt, Smith & Vance (1971). Statistical analysis of the results was carried out using Students' *t* test for paired or unpaired data.

Results

The haemodynamic effects of ORG6001

After the rapid intravenous injection of ORG6001 (1 or 10 mg/kg) into the right atrium, there were immediate, transient, and apparently dose-dependent, decreases in diastolic blood pressure and in left ventricular d*P*/d*t max* and increases in heart rate and in coronary blood flow. These immediate haemodynamic effects of ORG6001 are summarised in Table 1 and illustrated in Figure 1. However, 10 min after the intravenous injection,

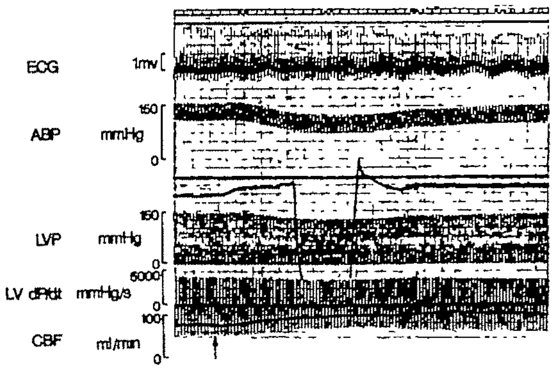


Figure 1 The immediate effects of an intravenous injection of ORG6001, 10 mg/kg (at arrow) on (from top to bottom), the electrocardiogram lead II (ECG), aortic blood pressure (ABP), left ventricular pressure (LVP), left ventricular d*P*/d*t* (LV d*P*/d*t*) and left circumflex coronary blood flow (CBF). The time marker at the top of the trace represents 1 s intervals

Table 1 The immediate (0-60s) haemodynamic effects of ORG6001 administered intravenously to anaesthetized greyhounds.

	ORG6001	
	1.0 mg/kg (n = 6)	*10 mg/kg (n = 9)*
Decrease in diastolic blood pressure (mmHg)	−10 ± 1 (8%)	−27 ± 7* (22%)
Increase in heart rate (beats/min)	+17 ± 8 (9%)	+11 ± 6 (6%)
Decrease in left ventricular d*P*/d*t max.* (mmHg/s)	−117 ± 36 (5%)	−218 ± 57 (9%)
Increase in coronary blood flow (ml/min)	+22 ± 5* (43%)	+31 ± 8* (39%)

Values are mean ± s.e.
* Change significant at $P < 0.05$ level.

Table 2 Haemodynamic changes induced by ORG6001, 10 min after intravenous administration in anaesthetized greyhounds.

	ORG6001			
	1.0 mg/kg		*10 mg/kg*	
	Before	*After*	*Before*	*After*
Mean blood pressure (mmHg)	145 ± 2	143 ± 1	143 ± 3	143 ± 3
Heart rate (beats/min)	185 ± 13	180 ± 12	183 ± 1	187 ± 7
Cardiac output (l/min)	3.4 ± 0.5	3.2 ± 0.4	4.3 ± 0.3	4.4 ± 0.4
External cardiac work (kg m min^{-1})	3.8 ± 0.6	3.7 ± 0.7	4.6 ± 0.4	4.5 ± 0.5
Left ventricular dP/dt max (mmHg/s)	3216 ± 421	3050 ± 376	2810 ± 141	2760 ± 141
LVEDP (mmHg)	7 ± 1	7 ± 1	8 ± 1	9 ± 1
Coronary blood flow (ml/min)	49 ± 2	52 ± 3	73 ± 7	83 ± 10
Myocardial oxygen consumption (ml/min)	8.4 ± 0.5	9.7 ± 0.9	9.8 ± 1.2	11.1 ± 1.6

Values are mean of 9 observations at each dose level ± s.e.

there were no significant changes in blood pressure, heart rate, cardiac output, left ventricular d*P*/d*t max.*, LVEDP, coronary blood flow, myocardial oxygen consumption and external cardiac work (Table 2). ORG6001, at either dose, did not affect coronary or peripheral vascular resistance or whole body oxygen consumption (Table 3).

Effects of ORG6001 on ventricular arrhythmias occurring in the immediate 30 min post-ligation period.

The results of this present investigation are illustrated in Figure 2 and are compared with previous results (Marshall & Parratt, 1974) obtained in control dogs and in dogs pretreated with lignocaine in Table 4. Ventricular arrhythmias are common in this preparation and include sustained runs of ventricular tachycardia (defined as more than 6 consecutive ventricular beats) particularly during the 16-20 min post-ligation period (Table 4 and Figure 2). Two out of the 4 dogs which received oral placebo fibrillated during this time. In contrast, significantly fewer arrhythmias were seen during the entire 30 min post-ligation period in dogs pretreated with ORG6001, administered intravenously in a dose of 10 mg/kg (15 min or 1.5 h before ligation) or orally, in a dose of 50 mg/kg, 4 h previously (Figure 2 and Table 4); ventricular tachycardia was not observed in any dog pretreated with ORG 6001. Results obtained in the dogs given ORG (1.0 mg/kg i.v.) 15 min before ligation were more equivocal. Although these animals had fewer arrhythmias than the controls, the difference was only significant in the first 20 min period. Overall, only 2 out of the 17 dogs pretreated with intravenous ORG6001 fibrillated in the first 30 min and none given the drug orally 4 h before ligation succumbed during this period. Further indication of the duration of ORG6001 is apparent from the protection afforded by 10 mg/kg intravenously administered 1.5 h before ligation. None of the 6 animals administered this dose died and the number of arrhythmias was significantly less than in the control, untreated, animals over the 30 min post-ligation period.

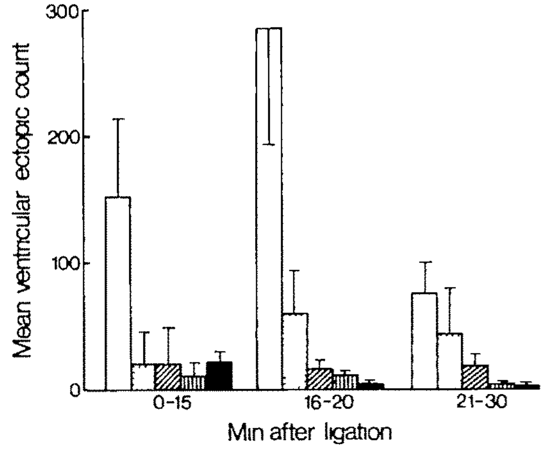


Figure 2 Mean ventricular ectopic counts in the immediate 30 min period after coronary artery ligation in control dogs and in dogs pre-treated with intravenously or orally administered ORG6001. The left hand set of histograms represents counts in the first 15 min, the middle set represents the crucial 16-20 min period and the right hand histograms represent the final 10 min period. Pre-treatment with ORG6001 suppressed the number of arrhythmias seen in any of these periods during the first 30 min after ligation. Columns (in each block of histograms) are, from left to right, control; ORG6001, 1 mg/kg 15 min i.v.; ORG6001, 10 mg/kg 1.5 h i.v.; ORG6001, 10 mg/kg 15 min i.v ; ORG6001, 50 mg/kg 4 h orally

Haemodynamic and metabolic consequences of ligation

The haemodynamic consequences of coronary artery ligation in this experimental model have been described in full elsewhere (Fisher *et al.*,

Table 3 Changes in vascular resistance and whole body oxygen handling before, and 10 min after, the intravenous administration of ORG6001 to anaesthetized greyhounds

	ORG6001			
	1.0 mg/kg (n = 6)		*10 mg/kg (n = 9)*	
	Before	*After*	*Before*	*After*
Peripheral vascular resistance (units)	44 ± 5	45 ± 6	36 ± 3	35 ± 3
Coronary vascular resistance (units)	24 ± 3	22 ± 2	20 ± 2	18 ± 2
Right atrial oxygen extraction (%)	11 ± 1	15 ± 3	12 ± 1	13 ± 2
Whole body oxygen consumption (ml/min)	107 ± 23	156 ± 52	148 ± 17	130 ± 13

Values are mean ± s.e.

Table 4 Analysis of the number of ventricular ectopic beats at various times after the acute ligation of the anterior descending branch of the left coronary artery in control (untreated) dogs and in dogs pretreated with lignocaine, ORG6001 or placebo

Group	*0-5 min*	*6-10 min*	*11-15 min*	*16-20 min*	*21-25 min*	*26-30 min*	*TOTAL*	*Deaths within 30 min*
Control	120 ± 42	150 ± 60	94 ± 67	286 ± 92	96 ± 38	52 ± 21	904 ± 260 (193-2250)	4/12
Lignocaine (2 mg/kg i.v.) 10 min pre-ligation	129 ± 61	201 ± 78	77 ± 73	283 ± 124	86 ± 32	3 ± 1*	813 ± 378 (104-1605)	2/16
ORG6001 (10 mg/kg i.v.) 15 min pre-ligation	19 ± 9*	14 ± 8*	2 ± 2*	11 ± 2*	5 ± 2*	2 ± 2*	43 ± 13* (9-84)	1/6
ORG6001 (10 mg/kg i.v.) 1.5 h pre-ligation	5 ± 3*	47 ± 45*	14 ± 8*	16 ± 7*	17 ± 9*	19 ± 12	116 ± 43* (33-324)	0/6
ORG6001 (1.0 mg/kg i.v.) 15 min pre-ligation	27 ± 18	8 ± 12*	30 ± 18	59 ± 34	32 ± 24	56 ± 52	210 ± 132* (9-698)	1/5
ORG6001 (50 mg/kg) ORAL 4 h pre-ligation	28 ± 18	33 ± 21*	9 ± 5*	3 ± 2*	1 ± 1*	4 ± 3*	78 ± 29* (3-185)	0/7
Placebo ORAL 4 h pre-ligation	42 ± 28	98 ± 27	143 ± 80	264 ± 96	110 ± 40	46 ± 12	—	2/4

Values are mean ± s.e., range in brackets.
* $P < 0.025$, Significantly different from control.

1973; Marshall *et al.*, 1974). The haemodynamic effects of ligation in this series of dogs pre-treated with ORG6001 (Table 5) were essentially similar and included marked decreases in cardiac output and external cardiac work. These changes are secondary to a decreased cardiac contractility, as manifested by transient decreases in left ventricular d*P*/d*t* *max* with more sustained increases in LVEDP (Table 5).

When a major branch of the left coronary artery is ligated, metabolic changes occur in the venous blood draining the ischaemic region which do not occur in blood draining the remaining, essentially normal areas. These changes in the control dogs have been described in detail by Marshall *et al.*, (1974) and include reductions in coronary venous $P\text{O}_2$, pxygen content, pH and lactate extraction and an increase in $P\text{CO}_2$. The administration of intravenous or oral ORG6001 before coronary artery ligation modified these metabolic consequences of myocardial ischaemia (Table 6). There were only slight, and insignificant, reductions in coronary vein $P\text{O}_2$ and oxygen content and the increase in $P\text{CO}_2$ and decrease in pH appeared to be less marked than in the control series previously described (Marshall *et al.*, 1974) The effects of ORG6001 on lactate handling by the ischaemic myocardium were especially striking and for the high intravenous dose (10 mg/kg 15 min before ligation) are summarized in Figure 3. None of the five dogs administered this dose of ORG6001, were producing lactate 30 min after ligation. This is to be compared with 5 out of the 8 dogs that produced lactate at this time in the control series. Myocardial lactate extraction in the ischaemic myocardium decreased slightly in the ORG6001 treated dogs (from a mean of +32% to a mean of +13%; $P > 0.05$) but changed significantly from extraction (+41%) to production (−8%) in the control, untreated dogs ($P < 0.025$). There were insignificant changes in lactate extraction by the normal myocardium following coronary artery ligation in either control or ORG6001 treated animals (Figure 3). Thus ORG6001 (10 mg/kg, i.v.) appeared to reduce the shift from aerobic (lactate extraction) to anaerobic metabolism (lactate production) in the acutely ischaemic zone of myocardium. Similar results were obtained in the dogs pretreated orally with ORG6001 (50 mg/kg). Before ligation both areas of myocardium extracted lactate to the same extent (coronary sinus 39 ± 6%, coronary vein 40 ± 5%). Thirty min after ligation lactate extraction in the normal region remained unchanged (34 ± 6%) and although in 2 out of 7 dogs, lactate production was evident in the ischaemic zone, the mean extraction value (19 ± 10%) was not significantly different from the pre-ligation control.

The lactate results in the dogs given ORG6001 (10 mg/kg) 1.5 h before ligation showed that 2 out of 5 dogs were producing lactate after 30 min, the mean change in extraction was from 32 ± 6% pre-ligation to 1 ± 9%, 30 min post-ligation ($P < 0.025$). The corresponding lactate extractions in the normal myocardium (assessed by coronary sinus sampling) were 33 ± 5 and 37 ± 6% respectively. The smaller dose of ORG6001 (1 mg/kg) given 15 min before ligation also had no effect on lactate production in the ischaemic myocardium. Two of the 5 dogs were producing lactate after 30 min ischaemia and the mean change (from 40 ± 4% to 9 ± 11%) was significant ($P < 0.025$) and similar to the change observed in the control series.

The effects of ORG6001 on potassium efflux from the ischaemic myocardium

An examination of the effects of ORG6001 on potassium efflux from the ischaemic myocardium was undertaken using simultaneous analysis of blood from the coronary vein (draining the ischaemic region) and from the coronary sinus (draining the essentially normal regions of the myocardium) As far as we are aware, there have been no previous studies on the effects of an antiarrhythmic drug on potassium efflux into a local vein draining an ischaemic region.

In the control series (no drug treatment) clear evidence was obtained of K^+ efflux into the local coronary vein. Before coronary artery ligation the

Table 5 The haemodynamic effects of acute coronary artery ligation in control dogs and in dogs pretreated with ORG6001

		Cardiac output (l/min)		*Left ventricular dP/dt max (mmHg/s)*		*LVEDP (mmHg)*	
	n	*Pre-ligation*	*30 min post ligation*	*Pre-ligation*	*30 min post ligation*	*Pre-ligation*	*30 min post ligation*
CONTROL	8	3.2 ± 0.4	2.2 ± 0.2*	2910 ± 280	2600 ± 190	6 ± 1	11 ± 2*
ORG6001 (10 mg/kg i.v.) 15 min pre-ligation	5	4.2 ± 0.2	3.5 ± 0.3*	3100 ± 193	2700 ± 114*	8 ± 1	10 ± 2
ORG6001 (10 mg/kg i.v) 1.5 h pre-ligation	6	2.9 ± 0.4	2.4 ± 0.3*	2833 ± 487	2683 ± 490	7 ± 2	10 ± 3*
ORG6001 (1.0 mg/kg i.v.) 15 min pre-ligation	4	3.5 ± 0.6	2.6 ± 0.1*	3025 ± 388	2700 ± 363	7 ± 2	9 ± 3
ORG6001 (50 mg/kg) ORAL 4 h pre-ligation	7	4.1 ± 0.4	3.5 ± 0.3*	3014 ± 380	2886 ± 359	11 ± 2	14 ± 2*

Values are mean ± s.e.
* $P < 0.05$ Paired 't' test.

Table 6 Changes in coronary vein oxygen extraction, $P\text{CO}_2$ and pH before and 30 min after, coronary artery ligation in control dogs and in dogs treated with ORG6001.

	Oxygen extraction %		*$P\text{CO}_2$ (mmHg)*		*pH (Units)*	
	Pre-ligation	*Post ligation*	*Pre-ligation*	*Post ligation*	*Pre-ligation*	*Post ligation*
CONTROL	54 ± 3	69 ± 3*	45 ± 2	60 ± 3*	7.347 ± 0.014	7.265 ± 0.023*
ORG6001 (10 mg/kg i v.) 15 min pre-ligation	49 ± 3	61 ± 4*	58 ± 2	71 ± 4*	7.310 ± 0.011	7.274 ± 0.009*
ORG6001 (10 mg/kg i.v.) 1.5 h pre-ligation	48 ± 3	52 ± 2	57 ± 6	62 ± 8	7.312 ± 0.048	7.282 ± 0.062
ORG6001 (1.0 mg/kg i.v.) 15 min pre-ligation	51 ± 4	52 ± 4	52 ± 2	57 ± 3	7.318 ± 0.031	7.278 ± 0.030
ORG6001 (50 mg/kg) ORAL 4 h pre-ligation	55 ± 2	63 ± 3*	59 ± 4	65 ± 4	7.326 ± 0.014	7.309 ± 0.010

Values are mean ± s.e.
* $P < 0.05$.

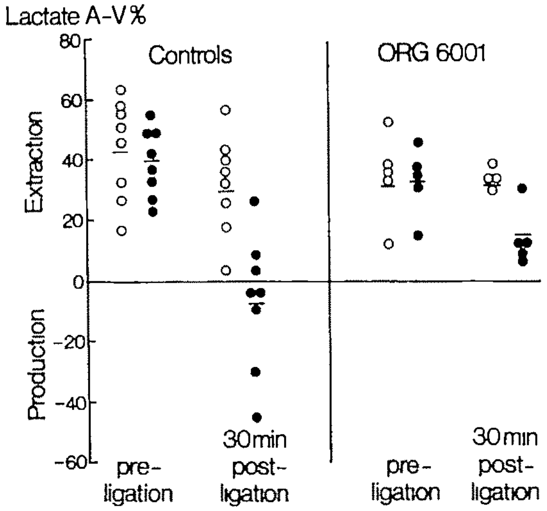


Figure 3 The effects of coronary artery ligation on the extraction of lactate by both normal (o, coronary sinus) and acutely ischaemic (•, coronary vein) regions of the myocardium in control dogs and in dogs given ORG6001 (10 mg/kg i.v.) 15 min before ligation. Mean values are represented by the bars. Lactate production in the ischaemic zone only occurred in the control dogs * $P < 0.025$.

arterial, coronary sinus and local coronary vein potassium values were similar (3.7 ± 0.1, 3.5 ± 0.2 and 3.5 ± 0.1 mEq/l respectively). Samples from these sites 30 min after coronary artery ligation gave values of 3.6 ± 0.2, 3.5 ± 0.2 and 4.3 ± 0.3 mEq/l respectively, $n = 12$). There was thus a substantial and significant ($P < 0.05$) efflux of potassium into the local coronary vein (i.e. from 3.5 ± 0.1 to 4.3 ± 0.3 mEq/l). It should be noted that potassium efflux into the coronary sinus was not observed in these control experiments and this result again clearly demonstrates the value of local venous sampling in metabolic studies on myocardial ischaemia.

No significant potassium efflux into the local coronary vein was observed after the intravenous administration of ORG6001 (1 mg/kg or 10 mg/kg 15 min before ligation); the coronary vein values were 3.5 ± 0.2 (pre-ligation) and 3.6 ± 0.2 mEq/l (30 min post ligation) after the lower dose of ORG6001 and 3.1 ± 0.4 and 3.5 ± 0.5 mEq/l respectively at the 10 mg/kg dose level. Similar results were obtained in the group of dogs given oral ORG6001 (50 mg/kg). Immediately prior to ligation, the arterial, local coronary vein and coronary sinus potassium levels were 3.5 ± 0.1, 3.6 ± 0.1 and 3.4 ± 0.3 mEq/l respectively. These levels remained unchanged 30 min after ligation,

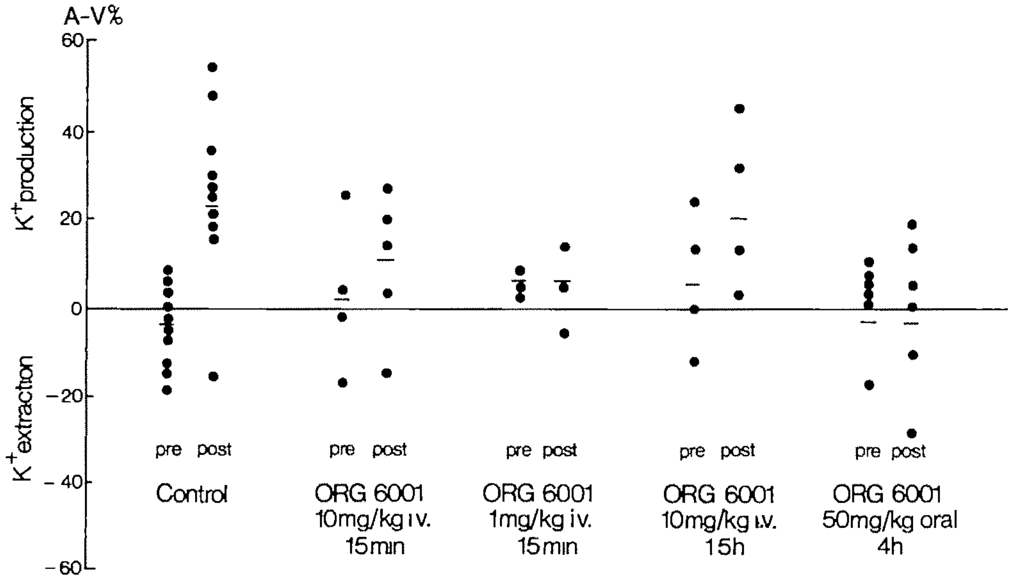


Figure 4 The effects of coronary artery ligation on potassium efflux into a local coronary vein in control dogs and in dogs pretreated with intravenously or orally administered ORG6001. Mean values are represented by the bars. Intravenous or oral ORG6001 prevented the marked efflux of potassium which was evident in control dogs, 30 min after ligation. * $P < 0.025$.

being 3.5 ± 0.1, 3.7 ± 0.1 and 3.4 ± 0.3 mEq/l respectively.

The potassium results are summarized in a rather different way in Figure 4 and it is clear that potassium efflux is evident 30 min after coronary artery ligation in the untreated dogs but not in those pretreated with intravenous or oral ORG6001.

Discussion

The most important reasons for the decrease in mortality of patients suffering from ischaemic heart disease have been the early detection and treatment of cardiac arrhythmias in the immediate period after myocardial infarction. Sixty-one per cent of deaths from myocardial infarction among patients younger than 65 occur within 1 h of onset (Gordon & Kannel, 1971), ventricular fibrillation being the terminal event in more than 90% of these early deaths (Pantridge & Adgey 1969). At present the most commonly used drugs for the treatment of these early ventricular arrhythmias are quinidine, procaineamide and lignocaine but there are drawbacks inherent in their use. Prominent side effects such as depression of myocardial contractility, hypotension and conduction abnormalities have especially been described with the use of quinidine and procaineamide (Jewitt, 1971; Mason, De Maria, Amsterdam, Zelias & Massumi, 1973). Lignocaine, the most effective of these three drugs, is thought not to produce marked haemodynamic effects (Austen & Moran, 1965) but suffers from the disadvantages that it is short acting (Harrison & Alderman, 1971), that it is not well absorbed orally, and most important, that it is less effective in the control of ventricular dysrhythmias that occur within 2 h of the onset of infarction, than those occurring later (Pantridge, 1971; Adgey, Allen, Geddes, James, Webb, Zaidi & Pantridge, 1971). There is thus a major need for an antiarrhythmic drug which produces minimal haemodynamic effects, which is effective against arrhythmias occurring in the early stages of infarction and which is reliably absorbed after oral administration. Such an orally active drug could then be given prophylactically to patients in whom the risk of a recurring myocardial infarct is great.

In these studies on experimental myocardial infarction, ORG6001 administered intravenously or orally, significantly reduced the number of ventricular ectopic beats in the first 30 min after acute coronary artery ligation. In addition, in contrast to the control animals, few of the treated animals succumbed to ventricular fibrillation during this period. The incidence of these early arrhythmias after acute coronary ligation depends critically on many factors which include the site of the ligature and size of the infarct produced (Thomas, Shulman & Opie, 1970), temperature, the anaesthetic used and the haemodynamic state of the animals. In these experiments, all ligations were made on the main intraventricular (anterior descending) coronary artery at a position 10-25 mm from the tip of the left atrial appendage and distal to the main septal branch. There was no difference in the size of ischaemic zone produced in the control dogs (21 ± 2% by weight of the free left ventricular wall) and in those treated with ORG6001 (23 ± 4%). In addition, control and treated animals did not differ immediately prior to ligation as regards oesophageal temperature (36.8 ± 0.6 and 37.7 ± 0.5°C respectively) haemodynamics (Table 5) or blood gases (Table 6).

Some indication of the duration of action of ORG6001 was given by the fact that, when administered intravenously 1.5 h before ligation, the drug was still capable of suppressing these early arrhythmias. Even more striking was the marked reduction in arrhythmias in dogs given ORG6001 (50 mg/kg) by mouth 4 h before ligation. Although this high oral dose of ORG6001 did not cause any obvious side-effects, it would be of interest to investigate the threshold oral dose of the drug which still exerted antiarrhythmic activity. Of relevance is the observation that two dogs given 25 mg/kg of ORG6001 by mouth, 4 h prior to ligation had respectively only 62 and 77 ventricular ectopic beats in the first 30 min after ligation. Reduction in the oral dose to 10 mg/kg in an additional two dogs did not markedly protect (218 and 320 ventricular ectopic beats respectively). In these studies, no attempt was made to elucidate the mode of action of ORG6001 but it is of note that the drug (2 mg/kg i.v.) is capable of reducing ventricular maximum following rate in anaesthetized dogs (Vargaftig *et al.*, 1975) and increases, in a dose-dependent manner, the effective refractory period of electrically driven guinea-pig atrial muscle in concentrations between 0.3 and 3.0 x 10^{-4} M (Marshall, unpublished observations). Previous work *in vivo*, also suggests that the antiarrhythmic properties of ORG6001 cannot be ascribed to either β-adrenoceptor blocking or local anaesthetic activity (Vargaftig *et al.*, 1975).

Although ORG6001, administered intravenously or orally, protected against these early ventricular arrhythmias, the drug did not affect the depression of myocardial contractility and cardiac output induced by coronary artery ligation. However the metabolic changes which occurred in the ischaemic myocardium were less marked in dogs treated with intravenous or oral ORG6001. One of the most marked metabolic changes which occurs in ischaemic cardiac tissue and which can be detected by selective coronary venous sampling is the production of lactate (Opie, Owen, Thomas & Samson, 1973, Marshall *et al.*, 1974). The normal heart is capable of extracting lactate (Figure 3). The discharge of lactate or decreased lactate uptake evident in the ischaemic myocardium is the result of increased glycolysis and a relative decrease in the rate of pyruvate entry into the Krebs cycle The stimulus for the increased glycolysis in ischaemia is probably both increased glucose uptake and glycogen breakdown. It is now widely accepted that in most cases, lactate production represents a shift from aerobic to anaerobic metabolism (Opie *et al*, 1973) and can be used as an index of impaired myocardial tissue oxygenation (Gorlin, 1972). Intravenous or oral ORG6001 largely prevented the occurrence of lactate production in the ischaemic zone of myocardium. Whether this is due to a direct beneficial effect of ORG6001 on blood flow or oxygenation in the ischaemic myocardium or whether it is secondary to the reduction in arrhythmias which themselves tend to decrease blood flow (Samet, 1973; Marshall *et al*, 1974) is at present unknown.

Pretreatment with ORG6001 also prevented the efflux of potassium ions into coronary venous blood. The relevance of the potassium ion to the occurrence of lethal ventricular arhythmias soon after the production of acute myocardial ischaemia has been the subject of a number of experimental studies. Some authors have deduced a causal relationship between cumulative potassium loss and the incidence of ventricular arrhythmias in the early stages of experimental myocardial infarction (Harris, Bisteni, Russell, Brigham & Firestone, 1954; Regan, Harman, Lehan, Burke & Oldewurtel, 1967) while others have failed to show any relationship (Wexler & Patt, 1960; Thomas *et al.*, 1970).

Although in this study only one estimation of potassium was made after ligation (at 30 min), we attempted to correlate the total number of ventricular ectopic beats with the egress of potassium in individual animals. We found no correlation ($r = 0.223$) between potassium egress and ventricular ectopic activity and therefore cannot assign a direct arrhythmogenic role to potassium ions. On the other hand, there was a good correlation between potassium egress and lactate production ($r = 0.763$). This may be explained by the fact that the further decrease in blood flow within the ischaemic region caused by arrhythmias not only results in an increased shift to anaerobic metabolism but, either by increasing the permeability of the cell membrane or by inhibiting $Na^+ - K^+$ ATPase, also induces an increased egress of potassium ions from the ischaemic region.

In doses which significantly suppressed early ventricular arrhythmias, ORG6001 caused only slight and transient haemodynamic changes. The immediate effects comprised modest falls in diastolic blood pressure, left ventricular pressure and left ventricular dP/dt *max* accompanied by tachycardia (presumably reflex in nature) and increased coronary blood flow. Ten minutes after injection, all parameters had returned to control values. Thus there was no evidence that ORG6001, in doses up to 10 mg/kg i.v., caused lasting myocardial depression, an effect readily seen with quinidine and procaineamide at lower doses in our experimental model (Marshall & Parratt, unpublished) and in man (Jewitt, 1971). In addition, animals pretreated with a high oral dose (50 mg/kg) of ORG6001, 4 h previously, were, just prior to coronary artery ligation, haemodynamically similar to control dogs.

We conclude that the non-hormonal amino steroid, ORG6001, is effective when administered both intravenously and orally, in suppressing the early arrhythmias induced by acute one-stage coronary artery ligation in anaesthetised dogs. ORG6001 would seem to hold important advantages over presently used antiarrhythmic drugs in that it has a long duration of activity and does not cause myocardial depression in effective antiarrhythmic doses.

We should like to thank Messrs I. Douglas, K. Gorman, R. Thomson, R. Spence and S. Gray for their excellent technical assistance; Mr R. McAllister for carrying out the lactate and potassium measurements and Mr R. Thomson for arranging the double-blind trial. We are also grateful to Dr M. Sugrue, Organon Laboratories, Newhouse, Lanarkshire, for generous supplies of ORG6001.

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(Received April 25, 1975)

Br. J. Pharmac. (1975), 55, 369-379

CALCIUM AND PANCREATIC SECRETION-DYNAMICS OF SUBCELLULAR CALCIUM POOLS IN RESTING AND STIMULATED ACINAR CELLS

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1 Pulse-chase experiments were carried out on pancreatic tissue lobules incubated *in vitro*, with ^{45}Ca as the tracer, in order to shed some light on the functional significance of the calcium pools associated with the various cell organelles of the acinar cell, especially in relation to stimulus-secretion coupling.

2 The kinetics of tracer uptake and release which were observed in the intact lobules suggest the existence of a number of intracellular pools, whose rate of exchange is slower than that across the plasmalemma.

3 The various subcellular fractions accumulate the tracer in different amounts: some (rough microsomes and postmicrosomal supernatant) showed little radioactivity and some (smooth microsomes and zymogen granule membranes) were heavily labelled; mitochondria and zymogen granules showed intermediate values.

4 The fractions are heterogeneous also in relation to the time course of uptake and release of the tracer· in rough and smooth microsomes and, especially, in the postmicrosomal supernatant both rates were fast; zymogen granules and zymogen granule membranes showed slow rates of uptake and little release during chase, intermediate rates were found in mitochondria.

5 In agreement with previous findings we observed that in ^{45}Ca preloaded lobules, stimulation of secretion (brought about by the secretagogue polypeptide caerulein) results in an increase of the tracer release which seems to be due primarily to the rise of the intracellular concentration of free Ca^{2+} and to the consequent increase of the transmembrane Ca^{2+} efflux Among the cell fractions isolated from stimulated lobules only the mitochondria exhibited a significantly lower ^{45}Ca level relative to the unstimulated controls.

6 It is concluded that, of the organelle-bound calcium pools, that associated with the mitochondria might be involved in the regulation of the calcium-dependent functions, including stimulus-secretion coupling; the calcium associated with the zymogen granule content probably has a role in the architecture of the organelle and in the functionality of the pancreatic juice, while the calcium bound to the membrane of the granules might be concerned with the regulation of its permeability properties.

Introduction

Calcium is known to play a fundamental role in a number of functions of secretory cells, such as the intracellular packaging of secretion products (Berneis, Pletscher & Da Prada, 1969; Wallach & Schramm, 1971), the regulation of the membrane permeability (Rubin, 1970; Rasmussen, 1970; Matthews, 1970; Dean & Matthews, 1970, Case, 1973; Matthews, 1974) and the transduction of the stimulation into the specific secretory response (stimulus-secretion coupling) (Douglas & Rubin, 1961; Douglas & Poisner, 1963; Douglas, 1968; Rubin, 1970; Rasmussen, 1970; Case, 1973; Matthews, 1974; Thorn, 1974). In relation to this latter function, over the last few years it has become increasingly evident that the coupling is not necessarily dependent on a change of calcium permeability of the plasmalemma. Rather, alterations in the intracellular distribution of Ca^{2+} might be the relevant event in the release process (Milligan & Kraicer, 1971; Nielsen & Petersen,

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1972; Case & Clauson, 1973; Malaisse, 1973, Matthews, Petersen & Williams, 1973; Heisler, 1974; Matthews, 1974; Milligan & Kraicer, 1974). This interpretation is based on the assumption that calcium is compartmentalized within the secretory cells in a number of pools, whose size can be rapidly adjusted in relation to the functional activity. However, up to now this assumption has not been proven because only very few secretory systems have been studied in detail in order to elucidate the functional distribution of calcium within the cells (Borowitz, 1969; Rahwan, Borowitz & Miwa, 1973; Herman, Sato & Hales, 1973; Simpson & Spicer, 1974).

This present investigation has been carried out in order to shed some light on these processes in the acinar cells of the pancreas. In a previous paper (Clemente & Meldolesi, 1975) we showed that in these cells the calcium distribution is very heterogeneous since only a small proportion of the total store is localized in the soluble cytoplasm while most of it is associated with various cytoplasmic organelles, especially the zymogen granules, the smooth microsomes and the mitochondria, and with the plasmalemma. The investigation has now been extended by the use of pancreatic tissue lobules incubated *in vitro* in the presence of ^{45}Ca. In particular we have studied: (a) the extent and the rate of accumulation of the tracer in the different cell organelles and (b) the metabolic stability of the various calcium pools in the resting cell as well as after *in vitro* pharmacological stimulation of secretion.

Methods

The animals used were 450-550 g male albino guinea-pigs (gift of Sigurtà drug Co., Milan, Italy) fasted for 24 h with water given *ad libitum*. The pancreases were rapidly removed from the animals and immersed in ice-cold Krebs-Ringer bicarbonate solution (calcium concentration 2.54 mM), equilibrated at pH 7 4 with 95% O_2 and 5% CO_2 and containing a complete set of amino acids, glucose and 2 mg/ml of bovine serum albumin (incubation medium). Groups of tissue lobules, as intact as possible, were dissected out as described by Castle, Jamieson & Palade (1972), transferred to 100 ml conical flasks containing 10 ml of incubation medium and incubated at 37° C in a shaking bath under an atmosphere of 95% O_2 and 5% CO_2. The tracers used were $^{45}CaCl_2$, L-[4,5-^{3}H]-leucine and D-[1-^{3}H]-sorbitol. At the end of the labelling incubations the lobules were recovered by filtration, rinsed with large volumes of ice-cold non-radioactive incubation medium and resuspended for 1 min in 50 ml of the latter medium. In most experiments they were then incubated at 37° C in non-radioactive incubation medium (chase incubation). Detailed conditions of labelling and of chase incubation of the lobules are described in the figure legends.

At the end of the incubations the lobules were homogenized in 0.3 M sucrose and cell fractions were prepared according to the procedures described elsewhere (Meldolesi, Jamieson & Palade, 1971), modified as indicated by Clemente & Meldolesi (1975). The incubation media were dissolved in 10 ml of Packard Instagel and transferred to disposable plastic vials. When a separate assay of the protein-incorporated radioactivity was needed, the samples were precipitated at 4° C with 10% tricloroacetic acid (TCA) and washed as described previously (Meldolesi, 1974a). Aliquots of the TCA-soluble and insoluble fractions were dissolved in 10 ml of Packard Instagel. All samples were counted in a SL30 Intertechnique liquid scintillation spectrometer (Intertechnique, Plaisir, France). In single label counting the efficiency of counting ^{3}H and ^{45}Ca was over 40% and 80%, respectively, in double label counting, 25% and 60%. Corrections for quenching and spill were made by external standardization. Protein was determined according to Lowry, Rosebrough, Farr & Randall (1951) on TCA-precipitates dissolved in 1 N NaOH, with crystalline bovine serum albumin as standard.

Materials

The sources of most of the materials used are given by Meldolesi & Clemente (1975). L[4,5-^{3}H] leucine (sp. act. 32 mCi/μmol) and D[1-^{3}H] sorbitol (sp. act. 6.8 mCi/μmol) were purchased from New England Nuclear, Langen, Germany. Caerulein was the kind gift of the Farmitalia Laboratories for Basic Research, Milan, Italy.

Results

Kinetics of calcium uptake and release

The exchange of calcium in pancreatic acinar cells was estimated by determining the uptake and the release of ^{45}Ca in tissue lobules incubated *in vitro*. As can be seen in Figure 1, the uptake of the tracer proceeds at a relatively slow rate, so that after 15 min of incubation the tissue radioactivity exceeds by only a factor of approx 2.5 the contribution of the extracellular space. Thereafter the accumulation of the tracer continues, and almost reaches a plateau after approximately 180 minutes. If the results are recalculated on the basis on the calcium concentration of the guinea-pig

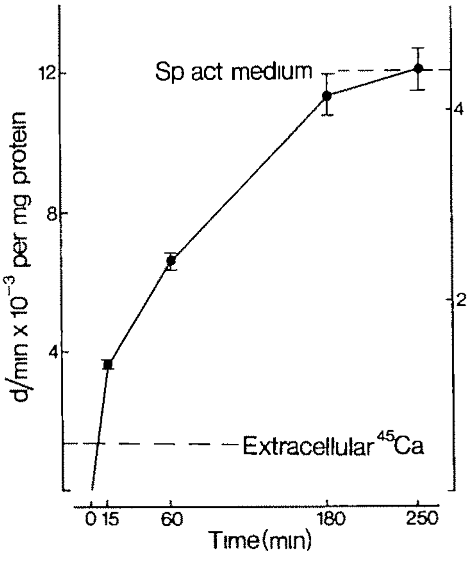

Figure 1 Time course of ^{45}Ca uptake in guinea-pig pancreas tissue lobules incubated for the time interval indicated on the abscissa scale in standard incubation medium containing 0.5 µCi of the tracer/ml. After incubation the lobules were thoroughly washed as described in the text but *not* incubated in chase medium. The ^{45}Ca content of the tissue is expressed as d/min per mg protein (left ordinate scale) as well as specific radioactivity (right ordinate scale) calculated on the basis of the calcium concentration of the pancreatic tissue reported previously (27.46 nmol/mg protein (Clemente & Meldolesi, 1975)) The extracellular calcium was calculated assuming a uniform distribution of the tracer in the extracellular compartment and knowing the size of the sorbitol space of the lobules, which amounts to 0.193 ml/g of tissue = 1 2 µl/mg protein. The latter values were determined in separate experiments in which comparable pancreatic tissue lobules were incubated for various intervals in incubation medium containing D-[1-^{3}H]-sorbitol (2 µCi/ml) (not shown in figures). The values are the mean of 4 experiments; vertical lines show s.e. mean.

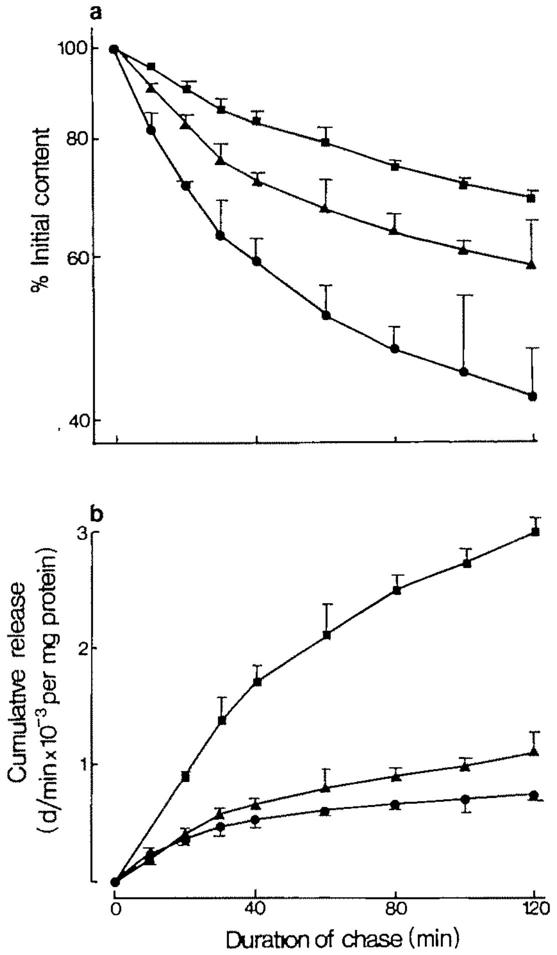

Figure 2 Effect of the length of the time of labelling with ^{45}Ca (0.5 µCi/ml) on the release of the tracer from pancreas tissue lobules during chase incubation. The data are plotted as (a) % calcium content of the tissue and (b) as cumulative release. Lobules obtained from the same animal were labelled for either 15 (●), 40 (▲) or 180 (■) minutes. After washing as described in the text the lobules were sequentially transferred to flasks containing chase medium with changes occurring after 5, 15, 25, 35, 45, 65, 85, 105 and 125 minutes. The radioactivity released during the first 5 min of chase incubation is not included in the results because it was considered to be primarily of extracellular origin. The values shown are the mean of 6 experiments; vertical lines show s.e. mean.

pancreas (27.46 nmol/mg protein (Clemente & Meldolesi, 1975), Figure 1) it becomes clear that this plateau corresponds to an almost complete equilibration of the intra and extracellular calcium pools, as suggested by the similar specific radioactivity of the tissue and incubation medium.

The release experiments are summarized in Figure 2, in which the efflux of ^{45}Ca from the pancreas lobules loaded for different time intervals is plotted against time. Figure 2a shows the time course of the changes of the ^{45}Ca content of the lobules during washout in chase medium, expressed as a percentage of the initial content; Figure 2b shows the cumulative release from the incubated lobules. On the basis of our sorbitol space data (see legend to Figure 1) we calculated that the ^{45}Ca released during the first 5 min of

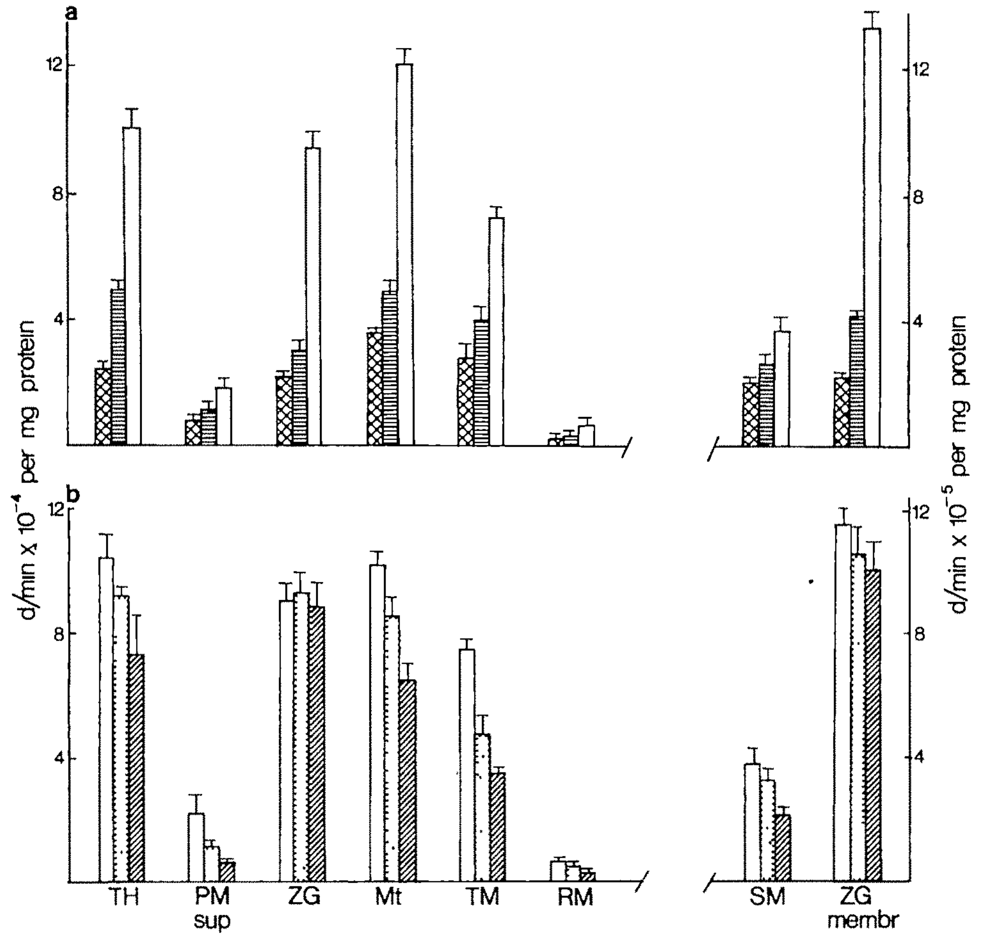

Figure 3 Distribution of ^{45}Ca in subcellular fractions isolated from pancreatic tissue lobules (a) effects of the length of the labelling time and (b) of the chase time. In the experiments summarized in (a) the lobules obtained from 3 animals were pooled, then divided into 3 sets which were labelled with ^{45}Ca (5 µCi/ml) for either 15 (double diagonally hatched columns) 60 (horizontally hatched columns) or 180 (open columns) min. At the end of the labelling incubation the lobules were thoroughly washed, incubated in chase medium for 5 min, washed again and then homogenized. In (b) all three sets of lobules were labelled with ^{45}Ca (5 µCi/ml) for 3 hours. At the end of the labelling incubation the lobules were thoroughly washed and incubated in chase medium for either 5 (open columns), 45 (stippled columns) or 120 (diagonally hatched columns) min (with changes of medium occurring after the first 5 min and every 10 min thereafter) before washing and homogenization. Cell fractions were isolated from the homogenates by differential and density gradient centrifugation. TH = total homogenate; PM Sup. = postmicrosomal supernate, ZG = zymogen granules; MT = mitochondria; TM, RM and SM = total, rough-surfaced and smooth-surfaced microsomes; ZG membr. = zymogen granule membranes. Results are mean of 3 experiments, vertical lines show s e mean.

chase incubation corresponds to the bulk of the extracellular radioactivity. As a consequence, the radioactivity released during this time was not included in the results, i.e. time zero of Figure 2 corresponds to the 5 min chase incubation and the ^{45}Ca radioactivity remaining in the tissue at a given time (Figure 2a) is expressed as a percentage of the total content of the lobules at that time. Moreover, the cumulative release data do not include the contribution of the 0-5 min chase medium. It is probable, therefore, that the results shown in Figure 2 are primarily due to the release of the tracer originating from intracellular sources. As can be seen in the figure, such an efflux proceeds at a relatively slow rate and is greatly dependent on the length of the labelling time:

proportionally, the release is slower in the lobules labelled for 40 min than in those labelled for 15 min, and is even slower in those labelled for 3 hours In all cases a constant slope is not obtained, even after 120 min of chase incubation.

Taken together, the results of the uptake and release experiments appear consistent with the idea that the intracellular calcium of the pancreas is distributed in a number of pools, whose rate of exchange is considerably slower than that across the plasmalemma. Analogous conclusions were reached previously by others (Case & Clausen, 1973; Matthews *et al.*, 1973, Heisler, 1974).

Experiments involving cell fractionation

The pancreatic homogenates obtained from tissue lobules exposed for various times to ^{45}Ca or incubated for various times in chase medium after prolonged labelling with the tracer were submitted to cell fractionation in order to estimate the kinetic features of the calcium pools associated with the different cell organelles. The characterization of the fractions used is reported in detail in previous publications (Meldolesi *et al.*, 1971; Clemente & Meldolesi, 1975). However, in the present investigation we have observed that the levels of radioactivity found in the crude mitochondrial pellet, isolated from labelled tissue lobules by differential centrifugation, remains practically unchanged after repeated resuspensions and reisolations (partially purified mitochondria). Thus, these two mitochondrial fractions are considered together in this paper.

The various fractions under study were found to accumulate the radioactivity in very different amounts (Figure 3). In particular, the rough-surfaced microsomes and the postmicrosomal supernatant are labelled to only a small extent during *in vitro* incubation of tissue lobules with ^{45}Ca, the zymogen granules and the mitochondria show levels of radioactivity comparable to those of the total homogenate, whereas the smooth-surfaced microsomes and, especially, the zymogen granule membranes show very high levels. Also in the time course of the uptake and release of the tracer a considerable heterogeneity was observed among the cell fractions. Some of them, such as the postmicrosomal supernatant, the rough and smooth-surfaced microsomes, accumulate the tracer during the labelling at a rate faster than that of the homogenate (Figure 3a). It is interesting to note that in these same fractions the rate of release of ^{45}Ca is also fast, as observed in the experiments reported in Figure 3b, in which the lobules were labelled first for 3 h (in order to reach an almost complete equilibration of all the different intracellular calcium pools with the incubation medium) and then reincubated up to 2 h in chase medium before cell fractionation. A different behaviour was observed in other fractions: the mitochondria have rates of uptake and release comparable with those of the homogenate; in zymogen granules and in their membranes the rate of accumulation is slow and there is little release during chase.

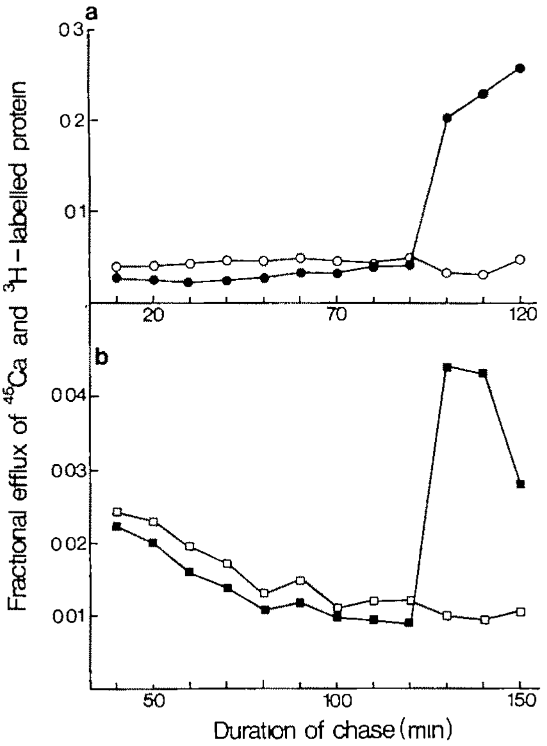

Figure 4 Effect of caerulein on the release of (a) ^{3}H-labelled proteins and (b) ^{45}Ca from pancreatic tissue lobules. The lobules were first labelled with ^{45}Ca (0.5 μCi/ml) for 60 min, then washed and reincubated in chase medium for the total time indicated on the abscissa scale of (b) with changes of medium occurring every 10 minutes. The medium used from the 20th to the 30th min of this incubation contained L-[4, 5-^{3}H]-leucine (5 μCi/ml). Caerulein (10^{-8} M) was added to half of the flasks (● and ■) during the last three 10 min intervals of incubation (120-150 min of chase incubation with respect to the end of ^{45}Ca labelling). The other flasks acted as controls (○ and □) At the end of the incubations the lobules were homogenized. Aliquots of the media and homogenates were analyzed for TCA insoluble ^{3}H and for ^{45}Ca radioactivity. The results are expressed as fractional efflux, i.e , as the ratio of the radioactivity released to the total radioactivity at each time point. The values shown are the average of 2 similar experiments.

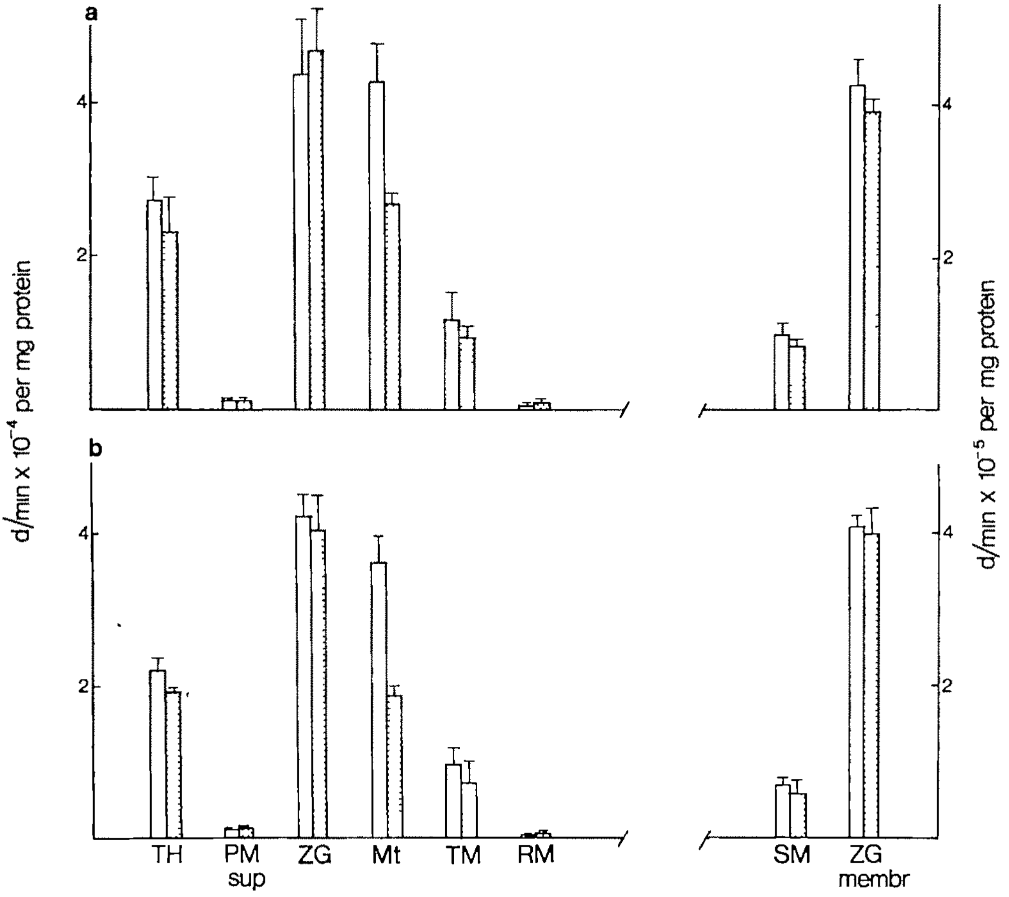

Figure 5 Effect of caerulein on the subcellular distribution of ^{45}Ca in pancreatic tissue lobules. Sets of lobules were labelled *in vitro* with ^{45}Ca (5 μCi/ml) for 60 min and then incubated in chase medium for a total of 120 min, with changes of medium occurring every 10 minutes Half of them were then incubated in non-radioactive medium with (stippled columns) or without (open columns) caerulein (10^{-8} M) for either (a) 5 min or (b) 30 minutes Stimulated and control lobules were washed, homogenized and fractionated by centrifugation TH = total homogenate; PM sup = postmicrosomal supernate; ZG = zymogen granules; Mt = mitochondria; TM, RM and SM = total, rough-surfaced and smooth-surfaced microsomes; ZG membr. = zymogen granule memebranes. The results shown are the mean of 3 experiments, vertical lines show s e. mean.

Experiments on pharmacologically stimulated lobules

It has been previously reported that, in preparations of rat pancrease incubated *in vitro*, the stimulation of secretion results not only in the release of the secretion products segregated within the zymogen granules, but also in an efflux of calcium from the acinar cells, most probably occurring through the plasmalemma (Case & Clausen, 1973; Matthews *et al.*, 1973; Heisler, 1974; Chandler & Williams, 1974). The existence of this phenomenon was verified in the pancreas of the guinea-pig by using tissue lobules first doubly labelled with ^{45}Ca and with L-[3,4-^{3}H]-leucine, and then incubated in chase medium long enough (a) to have the ^{3}H-labelled exportable proteins transported to the granules and (b) to minimize the variations of the fractional efflux of the ^{45}Ca. Finally these lobules were stimulated with caerulein, a polypeptide of known pancreozymin-like activity (Bertaccini, De Caro, Endean, Erspamer & Impicciatore, 1969; Meldolesi, 1970). As shown in Figure 4, the stimulation of secretion produces approximately a four-fold increase in the release of both the ^{45}Ca and the ^{3}H-labelled proteins. The efflux of ^{45}Ca cannot be due entirely to discharge by exocytosis, concomitantly with the proteins, because the ^{45}Ca/TCA-insoluble-^{3}H ratio observed in the medium is considerably (approximately four-fold) higher than that found in the zymogen granules isolated from doubly labelled pancreatic lobules (not shown in figures).

In order to investigate the intracellular events which are the basis of the increased calcium efflux triggered by the stimulation, we compared the distribution of the ^{45}Ca in subcellular fractions isolated from pancreatic lobules incubated for either 5 or 30 min with or without caerulein after *in vitro* loading with the tracer followed by chase incubation. As can be seen in Figure 5, no significant changes in radioactivity were found in the isolated fractions except for the mitochondria. In the latter fraction the exposure to the secretagogue drug produced approximately a 40% decrease which was evident 5 min after the onset of the treatment and persisted until the 30th minute.

Discussion

Calcium is known to play a critical role in all living cells because it is involved in the regulation of a number of fundamental functions, such as membrane permeability, enzyme activities, contractile processes, cell growth and adhesion. In secretory cells the importance of calcium is even greater because a compelling body of evidence, obtained in a number of different systems, indicates that a sudden rise of the concentration of free Ca^{2+} in the soluble cytoplasm is the common trigger of the secretory response (for reviews see Douglas, 1968; Rubin, 1970; Rasmussen, 1970, Matthews, 1970, 1974; Thorn, 1974). Such an increase in the free Ca^{2+} may occur because of an enhanced membrane permeability induced by the action of either the specific secretagogues (Douglas & Rubin, 1961, Douglas & Poisner, 1962; Douglas, 1968; Rubin, 1970) or calcium ionophores (Prince, Rasmussen & Berridge, 1973; Foreman, Mongar & Gomperts, 1973; Eimerl, Savion, Heichal & Selinger, 1974; Cochrane & Douglas, 1974; Kagayama & Douglas, 1974; Williams & Lee, 1974; Russel, Hansen & Thorn, 1974; Garçia, Kirpekar & Prat, 1975) as well as through a redistribution of the intracellular store, from the organelle-bound to the soluble pools. Recent experiments suggest that in a number of secretory systems, both endocrine and exocrine, including the pancreatic acinar cells, the latter mechanisms might account for the physiological regulation of secretion, i.e., that the free Ca^{2+} concentration of the soluble cytoplasm is controlled primarily by intracellular events (Milligan & Kraicer, 1971; Nielsen & Petersen, 1972; Case & Clausen, 1973; Matthews *et al.*, 1973; Malaisse, 1973, Milligan & Kraicer, 1974; Chandler & Williams, 1974). It is clear therefore that a detailed knowledge of the characteristics of the different intracellular calcium pools represents a necessary prerequisite to the understanding of the secretory process.

The information which is available on this problem has been mostly obtained by two different experimental approaches. On the one hand, the transcellular fluxes of calcium have been thoroughly studied in a number of secretory systems, under a variety of carefully controlled experimental conditions. The results obtained so far appear always consistent with the existence of a considerable degree of intracellular calcium compartmentalization (Milligan & Kraicer, 1971; Nielsen & Petersen, 1972; Case & Clausen, 1973; Matthews *et al.*, 1973, Malaisse, 1973; Milligan & Kraicer, 1974; Chandler & Williams, 1974). However, the direct identification of the various pools and the elucidation of their specific functional role is beyond the potentialities of these experiments.

Important results have also been obtained by investigating the *in vitro* binding ability of isolated subcellular structures (Poisner & Hava, 1970; Selinger, Naim & Lasser, 1970; Alonso, Bazerque, Arrigo & Tumilasci, 1971; Carafoli, 1973, Smith, Argent & Case, 1974). However, an obvious limitation of this type of study is that the data obtained are hard to correlate with the situation existing in the living cells, since the binding is greatly dependent on a number of conditions, such as the pH and the ionic strength of the suspending media, which in the *in vitro* experiments are selected arbitrarily, at least to a certain degree.

In principle, the approach that we have followed in the present investigation on the exocrine pancreas (and which had been followed previously by others working on the adrenal medulla; Borowitz, 1969), i.e. the study of well characterized subcellular fractions isolated from cells labelled with ^{45}Ca under controlled conditions, appears the most adequate to investigate directly some of the features and functions of the various calcium pools present in a secretory tissue. However, this approach too suffers a severe limitation because an extensive redistribution of the intracellular calcium is known to occur during homogenization and fractionation of the tissue. As discussed in detail in a previous paper (Clemente & Meldolesi, 1975), the quantitative evaluation of this type of artefact is laborious and sometimes not feasible. Thus, all our results should be considered with great caution.

However, we believe that our previous results (concerning the distribution of calcium in cell fractions isolated from fresh pancreas tissue; Clemente & Meldolesi, 1975) were much more affected by this type of artefact than those reported here. This conclusion is based on the fact that in the present cell fractionation experiments the bulk of the extracellular ^{45}Ca was removed from the lobules by a short incubation in chase medium, (see legend of Figure 2). Thus the influence of this source of artefact was greatly decreased. Moreover, the concentration of ^{45}Ca in subcellular structures is probably not greatly affected by the binding of the non-radioactive calcium present in small amounts in the solutions used. The fact that the binding artefacts do not have a major influence on our results is also indicated by the difference of ^{45}Ca distribution in cell fractions isolated from *in vitro* labelled pancreatic lobules (Figure 3, this paper) relative to the comparable fractions isolated from non-radioactive tissue homogenized in 0.3 M sucrose containing the tracer (Table 4, Clemente & Meldolesi, 1975). It should be noted that the fraction which is most heavily labelled in the latter condition, the rough-surfaced microsomal fraction, is the one which shows the lowest level of radioactivity when isolated from *in vitro*-labelled lobules; the converse is true for the mitochondria and zymogen granule membranes. Finally, the tracer associated with the fractions isolated from the loaded lobules is bound much more tightly than that adsorbed at the moment of homogenization. For instance, the partial purification of the crude mitochondria by repeated washes of the organelles did not appreciably change the radioactivity/protein ratio of the fraction isolated from the lobules but produced a considerable decrease of the same ratio in the fraction exposed to the tracer at the moment of homogenization (see Table 4, Clemente & Meldolesi, 1975). Analogous results were obtained with the zymogen granule membranes.

It seems reasonable to conclude, therefore, that, even if the results reported in the present paper are open to a degree of uncertainty, nevertheless they appear useful since they provide some further indication as to the distribution of calcium in the pancreatic acinar cells and permit some insight into the functional role of the different intracellular calcium pools.

In our work we have found that the concentration of calcium is relatively low in the post microsomal supernatant. for instance, after 3 h of *in vitro* incubation with ^{45}Ca the radioactivity recovered in this fraction/mg protein amounts to 1.8×10^4 d/min which is approximately one sixth of that of the total homogenate. If we assume that at this time the calcium of the supernatant is virtually in equilibrium with that of the incubation medium we can calculate the total calcium concentration of the fraction as approximately 150 nmol/g of tissue (These calculations are based on the corrected subcellular distribution of protein in the guinea-pig pancreas reported by Meldolesi, 1974b.) However, in all tissues so far investigated, the concentration of the free Ca^{2+} of the soluble cytoplasm has been found to be much lower, $10^5 - 10^8$ M (see Rasmussen, 1970; and Thorn, 1974; for reviews). Thus, it is most likely that only a small proportion of the calcium recovered in the post microsomal supernatant is accounted for by the free Ca^{2+}, whereas the bulk of it is contributed by calcium originally complexed with other molecules of the soluble cytoplasm or artefactually released from particulate pools. These considerations explain why there is little change in the ^{45}Ca radioactivity of the postmicrosomal supernatant after pharmacological stimulation of secretion, a condition in which the concentration of only the free Ca^{2+} is expected to rise considerably.

The calcium associated with the cytoplasmic organelles appears to be mostly divided among three different major pools: those associated with the mitochondria, with the Golgi complex (the major component of the pancreatic smooth-surfaced microsomes; Meldolesi *et al.*, 1971) and with the zymogen granules. Since the subcellular distribution of protein in the guinea-pig pancreas is known (Meldolesi, 1974b), it can be calculated that of the total ^{45}Ca taken up by the pancreatic lobules during 3 h of *in vitro* labelling approximately 17%, 20% and 30%, respectively, can be recovered in the corresponding fractions. Little calcium is probably associated in the living cell with the rough endoplasmic reticulum: probably the considerable calcium level found in the rough microsomes by direct assay (Clemente & Meldolesi, 1975) is an artefact, as suggested by the high labelling of that fraction in the adsorption experiments (Table 4, Clemente & Meldolesi, 1975) and by its very low labelling in the present experiments with tissue lobules.

The functional significance of the three major pools is probably quite different. Two of them (those associated with the mitochondria and smooth microsomes) appear to have relatively fast uptake and release properties, compatible with a direct involvement in the regulation of the Ca^{2+}-dependent cellular functions. In this respect it should be noticed that in other tissues the organelles recovered in theses two fractions have been found to be capable of energy-dependent calcium binding (Selinger *et al.*, 1970; Poisner & Hava, 1970; Alonso *et al.*, 1971; Carafoli, 1973).

Furthermore, among the various cell fractions investigated, the mitochondrial fraction was the only one which exhibited a significant decrease in ^{45}Ca early after pharmacological stimulation of secretion with caerulein. Even if the results obtained so far are not sufficient to exclude the contribution of the other cellular pools in the stimulus-secretion coupling, nevertheless they seem to represent the first direct indication that the Ca^{2+} involved in such a process might be primarily of mitochondrial origin. This conclusion seems to correlate well with the recent observation by Nishiyama & Petersen (1975) that in pancreatic acinar cells an increase in Na conductance at the level of the plasmalemma represents the primary event after exposure to secretagogue drugs, because a rise in Na^+ concentration of the medium is known to promote specifically the release of calcium from isolated mitochondria (Carafoli, 1973). Furthermore, it has been recently reported that *in vitro* treatment of pancreas preparations with drugs known to interfere with the mitochondrial calcium binding results in a stimulation of secretion (Smith *et al.*, 1974).

In contrast, the slow kinetics of ^{45}Ca accumulation in zymogen granules during tissue labelling and the stability of the granule-associated radioactivity during chase incubation, even in the presence of secretagogue drugs, strongly suggest that the calcium pool associated with this type of organelle is not directly in equilibrium with the rest of the intracellular calcium store. These findings further confirm the idea that in the pancreas, as previously suggested in other secretory systems (Berneis *et al*, 1969; Wallach & Schramm, 1971), the role of the calcium segregated within the secretion granules might be concerned not with the regulation of the cellular activity but with the architecture of the granules themselves and with the functionality of the released secretion products (Clemente & Meldolesi, 1975; Ceccarelli, Clemente & Meldolesi, 1975).

The association of large amounts of ^{45}Ca with the membranes of the zymogen granules deserves a special comment. Both the slow rate of accumulation and the stability during chase suggest that this pool is not involved in stimulus-secretion coupling. Rather, it might play an essentially structural role. In pancreatic acinar cells the membranes of zymogen granules are the membranes specifically capable of fusing with and being incorportated into the apical portion of the plasmalemma during exocytosis. In a variety of cell types the plasmalemma has been found to bind considerable amounts of calcium (Forstner & Manery, 1971; Perdue, 1971; Shlatz & Marinetti, 1972; Nijjar & Pritchard, 1973). From cytochemical evidence (Goodford & Wolowyck, 1972; Herman *et al.*, 1973) and from the results of kinetic experiments (Borle, 1969a,b), it appears that a large proportion of this calcium is associated with the outer surface of the plasmalemma, where it is assumed to produce significant changes in membrane organization and properties (Manery, 1966; Burger, Fuji & Hanahan, 1968, Romero & Whittam, 1971). This function of calcium is presumably related to the capacity of the cation to form complexes and to bridge adjacent negative charges thereby producing alterations in the physical state of membrane components which result in the stiffening of the structure and in the decrease of the permeability of the membrane itself (Hanahan, 1966, Burger *et al.*, 1968; Reynolds, 1972).

If one bears in mind that the large calcium pool associated with the zymogen granule membrane appears to be mostly localized at its inner face, i.e., at the face which after exocytosis becomes exposed at the outer surface of the cell (Clemente & Meldolesi, 1975), it is difficult to escape the suggestion that the granule membrane is equipped for becoming a temporary portion of the plasmalemma not only in relation to its specific protein and lipid composition (Meldolesi, De Camilli & Peluchetti, 1974) but also to its calcium 'coating'. Whether the high calcium binding capacity of the zymogen granule and plasma membranes are due to analogous molecular mechanisms is now under investigation.

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(Received April 28, 1975.
Revised June 24, 1975.)

Br. J. Pharmac. (1975), 55, 381-385

INHIBITION BY APOMORPHINE OF THE METOCLOPRAMIDE-INDUCED CATALEPSY AND INCREASE IN STRIATAL HOMOVANILLIC ACID CONTENT

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1 The mechanism of the cataleptic effect of metoclopramide was analyzed by using drugs which alter the activity of dopaminergic or cholinergic neurones or the content of γ-aminobutyric acid in the central nervous system of rats.

2 The cataleptic effect of metoclopramide (20 mg/kg) was antagonized by apomorphine (10 mg/kg) and by atropine (50 mg/kg). Aminooxyacetic acid (AOAA, 25-50 mg/kg) potentiated the catalepsy induced by metoclopramide (5 mg/kg).

3 Metoclopramide alone did not alter the rectal temperature of rats. It did not alter the AOAA-induced hypothermia, but it partially antagonized apomorphine-induced hypothermia.

4 Metoclopramide induced a six-fold increase in striatal homovanillic acid (HVA) concentration, but it did not change the dopamine or noradrenaline content in the brain of rats. Apomorphine decreased the striatal HVA concentration in control and in metoclopramide-treated rats. Atropine and AOAA did not alter the metoclopramide-induced increase in striatal HVA concentration.

5 The results suggest that metoclopramide produces catalepsy by blocking striatal dopamine receptors.

Introduction

The antiemetic compound, metoclopramide disturbs the extrapyramidal control of motor functions in man (Casteels-Van Daele, Jaeken, van der Schueren, Zimmerman & van den Bon, 1970) and in rats (Costall & Naylor, 1973). Lesions in caudate-putamen and globus pallidus reduce or abolish the cataleptic effect of metoclopramide in rats (Costall & Naylor, 1973). Moreover, in mice the metoclopramide-induced catalepsy is associated with a nearly five-fold elevation of striatal homovanillic acid (HVA) concentration (Ahtee & Buncombe, 1974). Therefore metoclopramide could act, as has been suggested for the neuroleptic compounds (Carlsson & Lindqvist, 1963; Andén, Roos & Werdinius, 1964), by directly blocking the striatal dopamine receptors; the receptor blockade could induce an increased production of dopamine through a neuronal feedback mechanism. In the present experiments the effects of drugs, which alter the activity of dopaminergic or cholinergic neurones or the brain concentration of γ-aminobutyric acid (GABA), on the metoclopramide-induced catalepsy and change in the striatal HVA content were studied. A preliminary communication of this work was given to the British Pharmacological Society (Ahtee, 1975).

Materials and Methods

Female Wistar rats, weighing 180-220 g, kept on a standard diet and tap water *ad libitum*, were used. During the experiments the rats were kept in individual cages at 20-22° C. Catalepsy was scored as described by Simon, Malatray & Boissier (1970) and by Cashin & Sutton (1973). Four tests were employed: 3 cm high rod, 9 cm high rod, parallel bars and vertical grid. Each test was scored from 0 to 2. The scores of the four tests were added. The rectal temperature was measured with an electric thermocouple (Ellab Instruments, Copenhagen).

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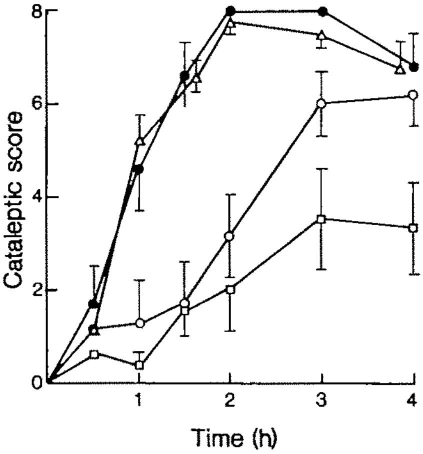

Figure 1 The cataleptic effect of metoclopramide (20 mg/kg) (s.c.) in rats pretreated with 0.9% NaCl solution (●), apomorphine (○), atropine (□), or aminooxyacetic acid (△). The times given were counted from the injection of metoclopramide. Apomorphine (10 mg/kg, s.c.) and atropine (50 mg/kg, i.p.) were given immediately before metoclopramide and aminooxyacetic acid (25 mg/kg, i.p.) was given 1 h before metoclopramide. Means from 6-8 rats are given; the vertical bars indicate the s.e. of the mean.

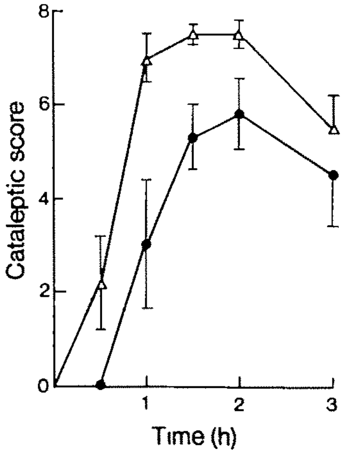

Figure 2 The cataleptic effect of metoclopramide (5 mg/kg) in rats pretreated with 0.9% NaCl solution (●) or aminooxyacetic acid (△). The times given were counted from the injection of metoclopramide. Aminooxyacetic acid (25 mg/kg, i.p.) was given 1 h before metoclopramide. Means from 6-8 rats are given; the vertical bars indicate the s.e. of the mean.

The rats were killed by decapitation, and the brain was rapidly removed and dissected. The striata from two brains were pooled for the estimation of HVA. The mean weight of tissue in each sample (striata from two rats) was 113 ± 23 mg (mean ± s.d., $n = 45$). HVA was estimated by the method of Portig, Sharman & Vogt (1968). In a few experiments the whole brain dopamine and noradrenaline contents were estimated by the method of Shellenberger & Gordon (1971). Recovery for added HVA was 69 ± 1% (mean ± s.e. mean from 5 estimations), for dopamine 89 ± 1.5% and for noradrenaline 86 ± 1.5% ($n = 4$). Results have been corrected for losses.

The drugs used were metoclopramide hydrochloride (a gift from H. Lundbeck & Co. A/S, Copenhagen), atropine sulphate and apomorphine hydrochloride (Pharmacopoea Nordica) and aminooxyacetic acid (AOAA) hemihydrochloride (Sigma Chemical Company, St Louis, Mo). Apomorphine was dissolved in 0.9% NaCl solution containing 0.1% sodium metabisulphite, AOAA was dissolved in 0.9% NaCl solution and neutralized with 1 N NaOH to pH 6; the other drugs were dissolved in 0.9% NaCl solution. The drugs were injected either s.c. or i.p. in a volume of 0.1 ml/100 g. The doses refer to the base or acid.

Results

Catalepsy and rectal temperature

Figure 1 shows that in rats metoclopramide (20 mg/kg) induced catalepsy which was maximal at 2-3 h after injection. Apomorphine (10 mg/kg) significantly reduced the cataleptic effect of metoclopramide at 1, 1.5 and 2 h after the metoclopramide injection ($P < 0.01$). Apomorphine alone induced stereotyped behaviour which consisted of sitting or standing in abnormal posture, continuous jerking of head or jaw upwards, backwards locomotion and continuous movement of one or both front legs as well as gnawing of the cage walls. The apomorphine plus metoclopramide-treated rats also showed some stereotypies during the first 2 h after drug injections. Atropine (50 mg/kg) significantly reduced the cataleptic effect of metoclopramide at 1 h ($P < 0.01$), 1.5 h ($P < 0.001$), 2 h ($P < 0.001$), 3 h ($P < 0.001$) and 4 h ($P < 0.01$) after injection.

Aminooxyacetic acid (25 mg/kg) given 1 h before metoclopramide did not change the cataleptic effect of metoclopramide (20 mg/kg; Figure 1). AOAA (25 or 50 mg/kg) alone did not induce catalepsy in the rats. The larger dose of AOAA made the rats fall into a sleep-like state from which they were easily aroused. This dose slightly accelerated the appearance of the

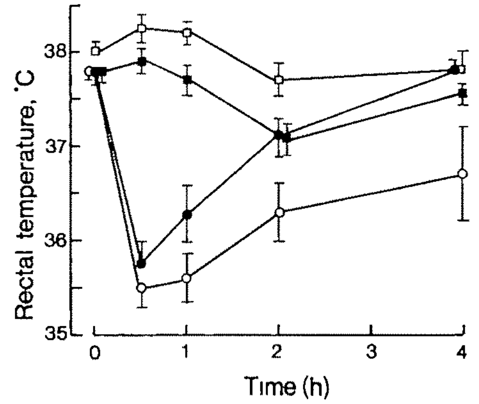

Figure 3 Rectal temperature in rats treated with 0.9% NaCl solution (□), 20 mg/kg of metoclopramide (■), 10 mg/kg of apomorphine (○) or with both metoclopramide and apomorphine (●). All drugs were injected s.c.; apomorphine was given immediately before metoclopramide. Times given were counted from the injection. Means from 6-8 rats are given; the vertical bars indicate the s.e. of the mean.

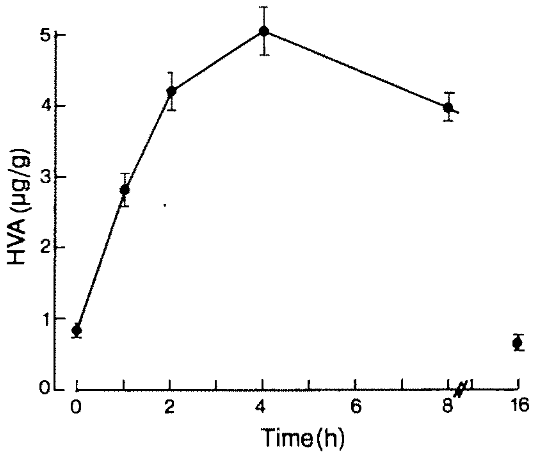

Figure 4 The effect of metoclopramide (20 mg/kg, s.c.) on the concentration of homovanillic acid (HVA) in the rat striatum. Means from 3-4 estimations (striata from two rats were pooled for each estimation) are given; the vertical bars indicate the s.e. of the mean.

cataleptic effect of metoclopramide (20 mg/kg). AOAA (25 mg/kg) pretreatment potentiated the cataleptic effect of metoclopramide (5 mg/kg) at 1 h ($P < 0.05$) and 1.5 h ($P < 0.02$) after the administration of metoclopramide (Figure 2).

Metoclopramide alone did not significantly change the rectal temperature of rats. Metoclopramide did not alter the AOAA-induced hypothermia, but it partially antagonized the apomorphine-induced hypothermia (Figure 3).

Striatal homovanillic acid content and cerebral dopamine and noradrenaline content

Metoclopramide (20 mg/kg) caused a six-fold elevation of striatal HVA content and the HVA content remained elevated at least until 8 h after the injection (Figure 4). The metoclopramide-induced increase of striatal HVA content was dose-dependent (Table 1). Two hours after the injection of metoclopramide (1.25 mg/kg) the

Table 1 Effect of metoclopramide on the striatal homovanillic acid (HVA) concentration in rats pretreated with 0.9% NaCl solution (saline), atropine or amino-oxyacetic acid (AOAA).

	Metoclopramide (mg/kg)	*HVA (μg/g)*		
		Saline	*Atropine*	*AOAA*
2 h*	0	0.84 ± 0.07	0.85 ± 0.02	0.93 ± 0.04
	1.25	1.53 ± 0.04	1.58 ± 0.18	1.73 ± 0.07
	5	3.62 ± 0.32	4.35 ± 0.21	3.74 ± 0.15
	20	4.21 ± 0.26	4.50 ± 0.38	4.71 ± 0.19
4 h*	0	0.75 ± 0.10	0.67 ± 0.11	0.82 ± 0.04
	1.25	0.81 ± 0.09	—	0.93 ± 0.08
	5	3.57 ± 0.23	—	3.94 ± 0.14
	20	5.04 ± 0.33	5.15 ± 0.29	5.80 ± 0.48

* The times given were counted from the injection of metoclopramide (s.c.). Atropine (50 mg/kg, i.p.) was given immediately and AOAA (25 mg/kg, i.p.) 1 h before metoclopramide. Means ± s.e. of the mean from 3-4 estimations are given (striata from two rats were pooled for each estimation).

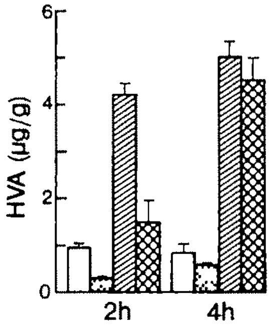

Figure 5 The effect of apomorphine (10 mg/kg, s.c.) on the metoclopramide (20 mg/kg, s.c.)-induced increase of the homovanillic acid (HVA) content in the rat striatum. The control rats (open column) were injected with 0.9% NaCl solution s.c., apomorphine (stippled) was given immediately before metoclopramide (hatched). Means from 3-4 estimations (striata from two rats were pooled for each estimation) are given; the vertical bars indicate the s.e. of the mean. (Cross hatching represents apomorphine + metoclopramide.)

striatal HVA content was doubled but after 4 h the striatal HVA content of metoclopramide-treated rats was normal again.

Table 1 shows that atropine alone did not cause a statistically significant decrease in the striatal HVA concentration, and it did not alter the metoclopramide-induced increase of striatal HVA concentration at 2 h or 4 h after the injection of the drugs. Atropine did not alter the metoclopramide-induced HVA increase at 1 h after the injections (metoclopramide, 20 mg/kg, alone = 2.80 ± 0.23 µg/g and metoclopramide + atropine = 2.72 ± 0.03 µg/g, mean ± s.e. mean, $n = 3$). As shown in Table 1, AOAA (25 mg/kg) did not alter the striatal HVA concentration in control or metoclopramide-treated rats. AOAA (50 mg/kg) did not alter the striatal HVA concentration in control rats or in rats treated with metoclopramide (20 mg/kg).

From Figure 5, it can be seen that 2 h after the injection of apomorphine (10 mg/kg) the striatal HVA concentration was decreased by 69% ($P < 0.001$). Two hours after the combined treatment with apomorphine and metoclopramide the striatal HVA concentration was 66% ($P < 0.001$) lower than after an injection of metoclopramide alone. Four hours after the injections of the drugs the effects of apomorphine had worn-off in both the control and metoclopramide-injected rats.

Metoclopramide did not change the concentration of dopamine or noradrenaline in the whole brain. The concentration of dopamine in the brain of control rats was 0.92 ± 0.05 µg/g (mean ± s.e. mean, $n = 3$), 3 h after an injection of metoclopramide (20 mg/kg) it was 0.83 ± 0.01 µg/g and 3 h after metoclopramide (40 mg/kg), 0.77 ± 0.04 µg/g. The brain noradrenaline concentrations of the same rats were 0.43 ± 0.02 µg/g (control), 0.42 ± 0.02 µg/g (metoclopramide, 20 mg/kg) and 0.41 ± 0.02 µg/g (metoclopramide, 40 mg/kg), respectively.

Discussion

Metoclopramide-induced catalepsy in rats was accompanied by a six-fold increase in striatal HVA concentration. This increased striatal HVA concentration without any decrease in brain dopamine concentration most probably indicates that dopamine formation is increased. Apomorphine, a drug which is thought to stimulate the striatal dopamine receptors (Ernst, 1967), reduced the catalepsy and the increase in striatal HVA concentration caused by metoclopramide. This antagonism lasted for about as long as the apomorphine stereotypies in control rats. Similarly to the dopamine receptor blocking neuroleptics (Fuxe & Sjöqvist, 1972; Kruk, 1972) metoclopramide partially antagonized the apomorphine-induced hypothermia. These findings support the suggestion that metoclopramide may block the striatal dopamine receptors.

The acetylcholine receptor blocking agent, atropine, clearly antagonized the metoclopramide-induced catalepsy but it did not antagonize the metoclopramide-induced increase of striatal HVA concentration. This is interesting because atropine antagonizes both the catalepsy and HVA increase produced by neuroleptic agents (O'Keeffe, Sharman & Vogt, 1970; Andén & Bédard, 1971). These results show that the prevention of drug-induced catalepsy by atropine does not simply restrain the overactivity of the dopaminergic neurones. Neuroleptic drugs and metoclopramide could have different effects on the pathways which mediate the feedback information controlling the activity of dopaminergic neurones.

Aminooxyacetic acid (AOAA), a compound which increases the cerebral GABA concentration (Wallach, 1961), antagonizes the effects of chlorpromazine and haloperidol on the striatal dopamine metabolism (Andén, 1974; Lahti & Losey, 1974). In contrast, in the present experiments AOAA did not alter the metoclopramide-induced increase in striatal HVA concentration. AOAA alone did not induce catalepsy in rats. However, it potentiated the metoclopramide-induced catalepsy. Whether this effect is associated with the AOAA-induced increase of the cerebral GABA content remains to be elucidated.

The present results suggest that metoclopramide, like the neuroleptic compounds, acts directly on the striatal dopamine receptors. However, biochemically and/or anatomically, the metoclopramide-induced catalepsy may differ from the catalepsy induced by neuroleptic compounds.

This work was supported by the Medical Research Council of the Academy of Finland. I would like to thank Mrs P. Olkkonen and Mr G. Buncombe for skilful assistance.

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(Received April 28, 1975)

Br J Pharmac. (1975), 55, 387-392

EFFECTS OF FASTING, STRESS AND DRUGS ON GASTRIC GLYCOPROTEIN SYNTHESIS IN THE RAT

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1 The relationship between gastric mucosal damage and synthesis of gastric glycoproteins, as measured by the rate of incorporation of N-acetyl-[^{3}H]glucosamine, was investigated in rats after fasting and restraint stress and a single administration of aspirin (200 mg/kg, orally), phenylbutazone (200 mg/kg, orally), prednisolone (200 mg/kg, orally), or adrenaline (2 mg/kg, i.p.). In one experiment, the effects of aspirin and phenylbutazone on carbohydrate content of the glycoproteins were also determined.

2 Restraint stress, phenylbutazone and aspirin resulted in acute gastric mucosal erosions in some of the rats. Adrenaline produced severe sub-mucosal haemorrhage, but no erosions or ulceration, while prednisolone and fasting gave no gross pathology.

3 The rate of incorporation of N-acetyl-[^{3}H]glucosamine into glycoproteins was decreased after all treatments except adrenaline. In the groups receiving restraint stress, aspirin or phenylbutazone, the decreases were more marked in rats which developed erosions than in those with no gastric pathology.

4 Aspirin and phenylbutazone also produced changes in the carbohydrate content of the glycoproteins, the effects again being greater in the rats which developed erosions.

5 The results are discussed in the context of a possible association between erosion formation and glycoprotein synthesis and it is proposed that inhibition of mucus glycoprotein biosynthesis may be one mode of action of stress and drugs in causing gastric mucosal damage.

Introduction

In studies of the pathogenesis of peptic ulcer, much attention has been concentrated on the aggressive forces of acid and pepsin and perhaps too little attention on the natural defence of the mucosa. The concept of a gastric mucosal barrier, protecting the stomach against its own secretions, was developed by Code and by Davenport (Code, Higgins, Moll, Orvis & Scholer, 1963; Davenport, 1964; Davenport, Warner & Code, 1964) and, although the precise location and nature of this barrier has not been fully defined, its function may be performed in part by the mucus coating the surface of the stomach and in part by the apical plasma membrane of the surface epithelial cells (Avery Jones, 1975). It is now generally accepted that the integrity of the mucus cells of the stomach and their ability to secrete mucus continuously are essential protective mechanisms (Kirsner, 1966) but the precise role of mucus in the aetiology of gastric ulcers and erosions is unknown.

The present study was undertaken to investigate the effects on rat gastric mucosa and mucus, of stress, phenylbutazone, aspirin, prednisolone and adrenaline, all of which are capable of producing gastric mucosal damage in the rat. As glycoproteins are the principal constituents of mucus (Kent, 1967; Spiro, 1970) the effects of stress and these drugs on the synthesis of gastric glycoproteins in the rat were studied as an index of mucus production and integrity. It is believed that, in mucus producing cells, the protein moiety of the mucus is produced by ribosomes associated with the endoplasmic reticulum and that membrane bound transferases probably attach one sugar at a time to the protein chain as it moves through the channels of the endoplasmic reticulum (Spiro, 1970). In the present study, the biosynthesis of the glycoproteins was assessed by measurement of the rate of incorporation of a radioactively-labelled sugar into rat gastric glycoproteins and by measurement of the carbohydrate content of the glycoprotein. In a previous study, N-acetyl-[^{3}H]glucosamine was found to give the best incorporation rates of a series of labelled hexoses and amino-sugars (Shillingford, Lindup & Parke, 1974) and was thus chosen for the present work. Some of these results have previously been communicated to the British and Italian Pharmacological Societies (Dekanski, MacDonald & Parke, 1974).

Methods

Female Biorex Wistar rats, 140-160 g, were used in all the experiments. Restraint was carried out according to the method of Brodie & Hanson (1960) in which the rats are wrapped in wire screening while under light ether anaesthesia and then allowed to recover from anaesthesia. The rats were restrained for 24 h at normal room temperature of 22°C, or for 24 or 6 h at a lower temperature (17°C). The rats were not fasted prior to the restraint period but had no access to food during restraint and therefore groups of rats fasted for the same length of time and at the same temperature as the restrained rats were included to determine the effects of fasting alone.

Assessment of mucosal damage

After the restraint period, or 6 h after drug administration, the rats were killed by cervical dislocation and the stomachs excised and washed in ice-cold 0.9% w/v NaCl solution (saline). The stomachs were examined and rated for pathology according to the following arbitrary scale: 0 = no damage; 1 = blood in lumen; 2 = pin point erosions; 3 = 1-5 small erosions (<2 mm); 4 = >5 small erosions; 5 = 1-3 large erosions (>2 mm); 6 = >3 large erosions.

Glycoprotein synthesis

The rate of incorporation of N-acetyl-[^{3}H]glucosamine into gastric glycoproteins was measured by minor modifications of the method of Lukie & Forstner (1972). Circular pieces of stomach from the corpus were removed using a cork borer, diameter 14 mm, to ensure similar sample sizes. Incubations were carried out at 37°C in a Mickle shaking incubator (60 rev/min). The samples were pre-incubated for 10 min in a modified Krebs Medium I (Lukie & Forstner, 1972) before the addition of 1 μCi of N-acetyl-O-[1-^{3}H]glucosamine (4 Ci/mmol, Radiochemical Centre, Amersham) and incubations were carried out for 2 5 h, gassing with 95% O_2 and 5% CO_2 every 20 minutes. Incubations were terminated by draining the medium and washing the samples twice with 5.0 ml of ice-cold saline. The tissue samples were then homogenized in 20 ml of 5 mM disodium ethylenediamine tetraacetate (EDTA; pH 7.4). Glycoproteins were precipitated overnight at 4°C with 10% trichloroacetic acid and 1% phosphotungstic acid. The acid-soluble supernatant was discarded and the precipitate washed twice with distilled water followed by two lipid extractions with chloroform/methanol (1 : 1, v/v). After air-drying, the precipitate was weighed and solubilised overnight in 2 ml of 0.5M NaOH. The samples were acidified before adding a 1 ml aliquot to 10 ml scintillant consisting of 3.3 g/l 2,5-diphenyloxazole and 0.2 g/l 1, 4-di-2-(4 methyl-5-phenyloxazolyl) benzene in Triton X-100/toluene (1 : 2, v/v). The tritium content was determined in a Packard Tricarb Model 3320 liquid scintillation spectrometer.

The carbohydrate content of the precipitated glycoprotein was determined in one experiment as follows: approximately 20 mg of each sample was weighed accurately and hydrolysed overnight in 2 ml of 2M HCl at 90°C. After hydrolysis, the samples were filtered and made up to 5 ml with distilled water. One ml of the filtrate was used for the combined estimation of fucose and hexoses by the method of Dische & Shettles (1948). A 3 ml aliquot of the filtrate was used for the assay of hexosamines by the method of Boas (1953) modified by the omission of the resin separation as similar values were obtained in preliminary experiments without this step. For sialic acid estimation 20-40 mg samples were hydrolysed for 1 h in 2 ml 0.025 M H_2SO_4 at 80°C. A 0.2 ml aliquot of the hydrolysate was assayed according to the method of Warren (1959).

Drugs

Phenylbutazone, aspirin and prednisolone were suspended in 0.1% aqueous Tween 80 and administered orally to rats via a stainless steel stomach tube at a dose of 200 mg/kg (0.5 ml/100 g bodyweight). (-)-Adrenaline bitartrate (BDH) was dissolved in saline and administered intraperitoneally to rats at a dose of 2 mg/kg (0.5 ml/100 g bodyweight). The animals were killed 6 h after a single administration

Results

The effects of fasting, restraint, cold and combinations of these treatments are given in Table 1. It was found that temperature markedly affected the degree of mucosal damage in the restraint experiments, the incidence and severity of erosions being far greater in the restraint plus cold group than with restraint alone for 24 hours. A reduction in the rate of incorporation of

Table 1 The effect of fasting, restraint and cold on the rate of incorporation of N-acetyl-[^{3}H] glucosamine into rat gastric glycoproteins

Group	*n*	*% With erosions*	*Mean erosion index*	*Incorporation of N-acetyl-[^{3}H] glucosamine d/min per mg glycoprotein*	*% Change from control value*
Control	9	0	0	1520 ± 200	–
Fasted 24 h	4	0	0	720 ± 240[1]	–53
Restrained 24 h	4	75	1.5	540 ± 170[2]	–65
Cold 24 h	10	0	0	1870 ± 320 NS	+24
Fasted plus cold 24 h	4	0	0	510 ± 130[2]	–67
Restrained plus cold 24 h	4	100	5.3	730 ± 300[1]	–52
Cold 6 h	6	0	0	1410 ± 420 NS	–8
Fasted plus cold 6 h	6	0	0	1110 ± 210 NS	–27
Restrained plus cold 6 h	6	50	1.8	640 ± 150[2]	–58

Portions of corpus tissue were incubated with 1.0 μCi N-acetyl-[^{3}H] glucosamine for 2.5 hours. Values shown are means for the number of rats (*n*) ± s.e. mean. Significance of difference from control value, Student's *t* test; (*P*), [1] <0.05; [2] <0.01; NS, not significant.

Table 2 The effect of various procedures on the rate of incorporation of N-acetyl-[^{3}H] glucosamine into gastric glycoproteins

Treatment	*n*	*% With erosions*	*Mean erosion index*	*Incorporation of N-acetyl-[^{3}H] glucosamine d/min per mg glycoprotein*	*% Change from control value*
Control	22	0	0	1470 ± 150	–
Restraint plus cold 6 h	11	55	1.9	890 ± 150[1]	–39
* Phenylbutazone (200 mg/kg orally)	11	37	0.9	910 ± 120[2]	–38
* Aspirin (200 mg/kg orally)	10	60	1.4	1180 ± 220 NS	–20
* Adrenaline (2 mg/kg i.p.)	6	0	0	1600 ± 320 NS	+9
* Prednisolone (200 mg/kg orally)	5	0	0	830 ± 310 NS	–43

* Rats were killed 6 h after single administration.
Values shown are means for the number of rats (*n*) ± s.e. mean. Significance of difference from control value, Student's *t* test; (*P*), [1] <0.05, [2] <0.01; NS, not significant.

N-acetyl-[^{3}H]glucosamine was also observed in the restrained rats but this was similar for the restraint and the restraint plus cold groups with no correlation between the extent of mucosal damage and the reduction in rate of incorporation. Fasting for 24 h produced a reduction in the rate of incorporation of N-acetyl-[^{3}H]glucosamine similar to that observed with 24 h restraint but without causing gastric mucosal damage The effect of fasting plus cold for 24 h on the rate of incorporation of N-acetyl-[^{3}H]glucosamine was slightly greater than that of fasting alone but not significantly so and again, no mucosal damage was observed. With a shorter experimental period of 6 h, cold alone or fasting plus cold produced no mucosal damage, whereas restraint plus cold for 6 h resulted in erosions in 3 out of 6 rats. The rate of incorporation of N-acetyl-[^{3}H]glucosamine was not significantly reduced after cold or fasting plus cold for 6 h, but was reduced after restraint plus cold for 6 hours.

In a separate series of experiments, the results of which are given in Table 2, restraint plus cold for 6 h, aspirin (200 mg/kg orally) and phenylbutazone (200 mg/kg orally), all resulted in acute gastric mucosal erosions in 37-60% of the treated rats. Adrenaline (2 mg/kg i.p.) produced severe sub-mucosal haemorrhage with no sign of discrete erosion or ulceration and the rats receiving prednisolone (200 mg/kg orally) showed no gross gastric pathology. The rate of incorporation of N-acetyl-[^{3}H]glucosamine was significantly reduced after restraint plus cold for 6 h and after phenylbutazone administration, but not after aspirin, adrenaline or prednisolone. In fact, in the prednisolone treated group, 3 out of 5 rats had markedly lowered rates of incorporation with 2 animals giving values in the normal range. The reduction failed to attain statistical significance owing to the large variation within the group.

If separate consideration is given to the rats which developed erosions (Table 3) then restraint

Table 3 Inhibition of the rate of incorporation of N-acetyl-[^{3}H]glucosamine into gastric glycoproteins in rats with and without gastric erosions

Treatment	*Erosions present*	*n*	*Incorporation of N-acetyl-[^{3}H]glucosamine d/min per mg glycoprotein*	*% Change from control value*
Control	NO	22	1470 ± 150	–
Restraint plus	NO	5	1130 ± 270 NS	−23
cold 6 h	YES	6	690 ± 150[3]	−53
* Phenylbutazone	NO	7	960 ± 170[1]	−34
(200 mg/kg orally)	YES	4	810 ± 160[2]	−45
* Aspirin	NO	4	1790 ± 320 NS	+22
(200 mg/kg orally)	YES	6	770 ± 150[2]	−48

* Rats were killed 6 h after a single administration.
Values shown are means for the number of rats (*n*) ± s.e. mean Significance of difference from control value, Student's *t* test; (*P*), [1] <0.05; [2] <0.01; [3] <0.001; NS, not significant.

Table 4 Effect of aspirin and phenylbutazone on the carbohydrate content of rat gastric glycoproteins

			Carbohydrate content nmol/mg glycoprotein			
Treatment	*Erosions present*	*n*	*Hexosamines*	*Hexoses*	*Fucose*	*Sialic acid*
Control	NO	8	33 ± 2	61 ± 12	14 ± 3	0.40 ± 0.03
* Aspirin	NO	3	30 ± 6 NS	46 ± 17 NS	20 ± 4 NS	0.42 ± 0.03 NS
(200 mg/kg orally)	YES	5	35 ± 3 NS	19 ± 4[2]	26 ± 2[2]	0.39 ± 0.01 NS
* Phenylbutazone	NO	4	41 ± 1[2]	21 ± 6[1]	21 ± 2 NS	0.39 ± 0.01 NS
(200 mg/kg orally)	YES	4	34 ± 3 NS	15 ± 3[2]	21 ± 3 NS	0.38 ± 0.01 NS

* Rats killed 6 h after single administration.
Values are means for the number of rats (*n*) ± s.e. mean. Significance of difference from control values, Student's *t* test; (*P*), [1]<0.05; [2]<0.01; NS, not significant.

plus cold for 6 h, phenylbutazone and aspirin all gave significant reductions in the rate of incorporation of N-acetyl-[^{3}H]glucosamine in rats with erosions. In the treated rats which failed to develop erosions, restraint plus cold for 6 h produced a small but non-significant reduction in N-acetyl-[^{3}H]glucosamine incorporation, while aspirin increased the incorporation but again not significantly so. A significant reduction in the rate of incorporation of the labelled hexosamine in rats without erosions was seen only in the group receiving phenylbutazone.

The results of a single experiment to determine the effects of aspirin and phenylbutazone on the carbohydrate content of rat gastric glycoproteins are given in Table 4. The changes in hexose and fucose content were greater in aspirin-treated rats with erosions than in those without. In the phenylbutazone-treated group the reduction in hexose content was greater in rats with erosions than in those without, but the reduction was significant for both groups. The fucose content of phenylbutazone-treated rats appeared similar in rats with or without erosions and, although perhaps slightly raised, failed to differ significantly from the control value. A significant rise in hexosamine content was also observed in the phenylbutazone treated group but only in the rats in which no erosions had formed. No changes in the content of sialic acids were observed.

Discussion

Decreases in the quantity, and carbohydrate content, of secreted mucus in rat and dog have previously been reported after administration of aspirin (Menguy & Masters, 1965), phenylbutazone (Menguy & Desbaillets, 1967) and prednisolone (Robert & Nezamis, 1963). Decreases in the glycoprotein content of rat and guinea pig gastric mucosa have also been shown after restraint stress (Ludwig & Lipkin, 1969; Lambert, Truchot, Andre & Chayvialle, 1971). These effects have led to the hypothesis that stress or drugs may cause gastric mucosal injury by inhibition of the biosynthesis of mucus components. In the present study, there are three theoretical possibilities regarding a relationship between erosion formation and the observed inhibition of glycoprotein synthesis. First, erosion formation and inhibition of glycoprotein synthesis are not related; second, impaired glycoprotein synthesis may be a direct result of mucosal cell damage; and third, mucosal pathology may be a consequence of impaired glycoprotein synthesis.

The first possibility is seen in fasting for 24 h which causes an inhibition in the rate of incorporation of N-acetyl-[^{3}H]glucosamine into gastric glycoproteins without causing gastric mucosal erosions. The incorporation also appeared to be lowered in some rats after prednisolone treatment, although no mucosal damage occurred, while adrenaline resulted in severe gastric pathology without any reduction in glycoprotein synthesis. However, it is known that more prolonged fasting (Robert, Bayer & Nezamis, 1963) or prednisolone treatment (Robert & Nezamis, 1963) does lead to mucosal damage and the observed reduction of glycoprotein synthesis may be a necessary prerequisite or even the cause of the damage. Also, the nature of the adrenaline-induced gastric damage was markedly different from that after treatment with the other agents used in this study. No discrete erosions or ulcerations were present but diffuse sub-mucosal haemorrhage affecting large areas of the stomach was observed. Thus, the primary effect of adrenaline appears to be on the blood vessels, causing vasoconstriction with no immediate effect on glycoprotein biosynthesis, although more prolonged treatment may lead to an inhibition of synthesis possibly through reduction of the mucosal blood supply.

The second possibility, that impaired glycoprotein synthesis is a result of mucosal damage is unlikely as first, the reduction in N-acetyl-[^{3}H]glucosamine incorporation can occur when no mucosal damage is present (e.g. after 24 h fasting and in phenylbutazone-treated rats which failed to develop erosions) and second, when mucosal damage does occur, the reduction in the rate of incorporation of N-acetyl-[^{3}H] glucosamine is not related to the intensity of the damage (e.g. restraint and restaint plus cold for 24 h, Table 1).

The third possibility, that mucosal damage is a consequence of impaired glycoprotein synthesis, may apply. We have shown that erosion formation after restraint plus cold stress, aspirin or phenylbutazone is associated with a reduction in the rate of incorporation of N-acetyl-[^{3}H] glucosamine into rat gastric glycoproteins. Changes in the carbohydrate content of the glycoproteins, also associated with erosion formation after treatment with aspirin or phenylbutazone, were also found. Several of the changes in the rate of incorporation of N-acetyl-[^{3}H]glucosamine and carbohydrate content occurred in treated rats which had not developed erosions suggesting that they not only accompany, but actually precede, erosion formation.

Thus it is possible that one mode of action of stress and drugs in causing gastric erosions is inhibition of the glycosylation of the mucus glycoprotein and hence a decreased synthesis of glycoprotein or the synthesis of a modified glycoprotein, or both. The changes in fucose and hexose content after treatment with aspirin and phenylbutazone suggest synthesis of a modified glycoprotein. A decreased synthesis of glycoproteins as measured by radioactive glucose uptake by sheep colonic mucosal scrapings in the presence of aspirin *in vitro* has previously been found (Kent & Allen, 1968) whereas the drug carbenoxolone has been shown to increase the rate of incorporation of radioactively labelled carbohydrates into glycoproteins of rat, ferret and human gastric mucosa (Shillingford, Lindup & Parke, 1974; Johnson, Lindup, Shillingford, Smith & Parke, 1975), an effect which may explain its beneficial effects on mucus production (Goodier, Horwich & Galloway, 1967) and its proven efficacy in the treatment of gastric ulcer (Sircus, 1972). Studies of the effects of carbenoxolone and analogues on the rate of incorporation of labelled carbohydrates into glycoproteins of rat gastric mucosa and their correlation with the prevention or healing of experimental ulcers or erosions are in progress, using a modified *in vitro* technique.

The authors are grateful to the directors of Biorex Laboratories Limited for the advice, encouragement and facilities provided.

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(Received May 5, 1975.)

Br. J Pharmac. (1975), 55, 393-402

THE USE OF RADIOACTIVE MICROSPHERES TO COMPARE THE EFFECTS OF HYDRALAZINE, GUANETHIDINE AND SK&F 24260 ON THE REDISTRIBUTION OF CARDIAC OUTPUT IN ANAESTHETIZED RABBITS

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1 The use of radioactive microspheres is described for the measurement of cardiac output in anaesthetized rabbits and its redistribution after the administration of drugs which lower blood pressure.

2 Hydralazine increased peripheral vascular conductance by 123% The vascular beds in which it had most effect were those of the carcass (mainly muscle) and the kidneys.

3 SK&F 24260, (1,4 dihydro-2, 6-dimethyl-4-(2-trifluormethylphenyl)-3,5,-pyridinedicarboxylic acid diethyl ester), had similar vasodilator actions. Its effect in the carcass contributed relatively more to the increase of total peripheral conductance. It also caused a remarkable degree of cerebral vasodilatation.

4 Guanethidine had a relatively small effect on total peripheral conductance and lowered blood pressure mainly by reducing stroke volume and cardiac output.

Introduction

Drugs used to lower arterial blood pressure may do so by interference with a variety of physiological mechanisms, and hence cause a redistribution of cardiac output which may differ qualitatively or quantitatively. Until recently it has been possible to study this redistribution only by the tedious assembly of information on blood flow to different organs or tissues from many separate experiments. The isotope-labelled microsphere method (Rudolph & Heymann, 1967), combined with direct sampling of arterial blood during microsphere injection (Jarai, 1969; Duncan, 1969), now makes it relatively easy to obtain a complete quantitative profile of blood flow to the major organs and tissues. Repeated injections may be made of γ-emitting isotopes whose energy spectra are sufficiently distinct.

In order to test the possibilities of this method for the analysis of the cardiovascular properties of drugs, it was decided to compare the effects of hydralazine, which acts by precapillary vasodilatation (Stunkard, Wertheimer & Redisch, 1954; Ablad, 1963) with those of SK&F 24260 (1,4 dihydro-2, 6-dimethyl-4-(2-trifluormethylphenyl)-3,5,-pyridinedicarboxylic acid diethyl ester) (Loev, Ehhreich & Tedeschi, 1972) with similar properties (Fielden, Owen & Taylor, 1974). Their circulatory effects are contrasted with those of the adrenergic neurone blocking drug, guanethidine.

Methods

Adult male Chinchilla rabbits weighing 2.0-3.9 kg (mean 2.97 ± 0.07 kg; s.e.) were anaesthetized with sodium pentobarbitone (Abbott Laboratories) 30 mg/kg intravenously. The trachea was cannulated and catheters were inserted into both femoral arteries, one for measuring arterial pressure and the other for blood sampling for cardiac output determination. The left ventricle was catheterized via the right carotid artery for the injection of microspheres. Arterial and ventricular pressures (1 mmHg = 1.333 mbar) were measured with strain gauge manometers (Devices, Ltd.), and were recorded on a Schwarzer Polygraph. Heart rate was recorded with a heart rate meter (Wyatt, 1957) actuated by the arterial pulses.

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The rabbit was placed in a supine position on a warm table, and the intensity of a light above it was adjusted to maintain the rectal temperature at 38.5° C. When the depth of anaesthesia was stable, arterial pH, $P\text{CO}_2$ and $P\text{O}_2$ were measured using 0.6 ml blood samples (Radiometer). The values were corrected for the difference between electrode and body temperatures. The method of measuring both organ blood flow and cardiac output by the use of radioactive microspheres was as described by Duncan (1969). The exact composition of these microspheres has not been released but the manufacturers describe them as 'carbonized plastic spheres of uniform size and shape'. The nominal diameter was 25 μm ± 5. Measurement of small samples from each batch showed the mean diameter in different batches ranged from 22 ± 5 μm to 27 ± 6 μm. The density of the microspheres is between 1.1 and 1.6 (mean 1.4) and when injected into the heart they are uniformly mixed with the blood. Approximately 60,000-100,000 25 μm microspheres (3M Company, Minnesota) labelled with ^{46}Sc and homgeneously suspended by ultrasonic agitation in 1 ml NaCl 0.9% w/v solution, containing a drop of Tween 80, were injected into the left ventricle from a polyethylene syringe. Withdrawal of blood from the femoral artery was begun at 5 ml/min about 10 s before the injection of the microspheres, and was continued throughout and for 15-20 s afterwards. The suspension of microspheres was injected over a period of 20-30 s, the syringe being continuously agitated to maintain an even dispersion. Fifteen to twenty minutes after the first injection of microspheres arterial pH and blood gas tensions were measured once more to ensure that the animal was in a normal physiological state, and the appropriate dose of either hydralazine (Apresoline, Ciba Laboratories) SK&F 24260 or guanethidine (Ismelin, Ciba Laboratories) was then given. Doses and routes of administration were chosen to produce a gradual fall in pressure over a 20-30 min period.

When the pressure had stabilized at a lower level the pH and blood gas tensions were measured again. A second injection of microspheres, labelled with ^{85}Sr, was given as described previously. The rabbits were then killed with an overdose of pentobarbitone.

The radioactivity of the whole animal was measured with a small total body γ-counter (Johnson & Warner, unpublished observations). The animal was then dissected and various organs were removed and weighed. The radioactivities of the large organs, hind legs, skin, large and small intestines, stomach and liver, and of the carcass were measured in the total body counter. The hind limbs were measured rather than the fore limbs since they are larger and contain more skeletal muscle. Although both femoral arteries were tied the collateral circulation was sufficient to give a normal blood flow; not only was there a good correlation in flows between left and right leg, but statistically significant changes in flow after administration of guanethidine and SK&F 24260 were seen. Smaller organs and blood samples were placed in vials and counted in an automatic well-type scintillation counter (Phillips P.W. 4520/00S). The design of this counter had been modified so that the geometrical error of counting with varying position within the vial was less than 5% (Bruce, N.W., Johnson, P. & Wyatt, D.G., personal communication).

The errors due to self-absorption and varying geometry, when counting organs or whole animals of different sizes in the small total body counter, were calculated by injection of ^{46}Sc or ^{85}Sr microspheres of measured radioactivity into rats, guinea-pigs or rabbits of different weights (25 g-4 kg) which were then counted. The necessary corrections (varying as negative exponentials with weight) were incorporated into a computer programme, which also introduced corrections for the overlap of Sc counts on the Sr channel in each counter, for the different sensitivities of the two counters and for variations in background counts. This was used to calculate from raw data the corrected cardiac output, organ blood flows in ml/min and ml/min^{-1} 100 g^{-1} and vascular resistances.

Cardiac output was calculated as

$$\frac{R_{Total}}{R_{Femoral}} \times Q_F$$

where Q_F is the rate of flow into the femoral syringe (5 ml/min) and R_{Total} and $R_{Femoral}$ are the total radioactivity injected and the radioactivity of the femoral syringe respectively. The blood flows to individual organs were calculated in a similar fashion. Vascular peripheral resistances were calculated, in arbitrary units, as

$$\frac{\text{arterial pressure (mmHg)} \times \text{body weight (kg)}}{\text{blood flow (ml/min)}}$$

'Conductance' is the reciprocal of resistance. All results are expressed as mean ± s.e. mean. The limits for the differences between the means were calculated using a paired Student's t-test.

Validation

Sufficient microspheres were injected to ensure that every organ examined received at least 400 (Buckberg, Luck, Payne, Hoffman, Archie & Fixler, 1971) and that the radioactivity of each organ was several times the background count in whichever detector it was measured. The numbers injected, therefore, were increased to take account of the isotopic decay of the stock microsphere suspension.

The mean calculated proportion of total injected radioactivity arrested in the lungs was 3.9 ± 0.4% (s.e.). This must have included bronchial arterial flow. The relatively low proportion indicates that most microspheres were trapped in the first capillary bed downstream from the site of injection. The homogeneity of microsphere distribution in the circulation was demonstrated by simultaneous estimations of cardiac output from femoral and brachial arterial samples ($r = 0.97$, $n = 6$), and by the close correlation between blood flows to right and left kidneys ($r = 0.981$, $n = 63$). There was also a highly significant correlation between flows to all organs estimated after simultaneous injection of ^{46}Sc and ^{85}Sr labelled microspheres. Hence the number of microspheres used and the femoral arterial sampling rate were adequate to provide reliable estimates of blood flows.

Occasionally, in about 5% of rabbits, injection of microspheres caused an immediate large fall of heart rate and blood pressure presumably attributable to coronary infarction; such experiments have been excluded from these results.

Results

Table 1 shows the changes in cardiac output and organ blood flows in 12 rabbits in which microspheres labelled with different isotopes were injected on two occasions at 30-45 min intervals without drug administration. Only mean arterial pressure and blood flow to the stomach fell significantly. These observations agree with those of Duncan (1969) and Leduc (1972) who found that the injection of microspheres 30-45 min apart in anaesthetized pregnant rabbits had little effect on cardiac output or its distribution. There were no significant changes in blood gas values, heart rate, or total peripheral resistance. The fall in arterial pressure is attributed to the effect of anaesthesia and time; a similar decrease is seen in anaesthetized rabbits which have not been given microspheres.

The relative proportion of organ or tissue to body weight in adult rabbits is approximately constant. Hence for economy of space organ weights are only given in Table 4 (observations on guanethidine), in which group of experiments measurement of organ weights was most detailed.

Table 1 Control observations (no drug given) on two microsphere injections 30-45 min apart on 12 rabbits

	^{46}Sc	^{85}Sr
Cardiac output (ml kg^{-1} min^{-1})	133.6 ± 8.7	129.3 ± 8.9
Arterial pressure (mmHg)	98 ± 2.9	89 ± 2.6*
Heart rate (beats/min)	320 ± 11	335 ± 13
Total peripheral resistance	0.771 ± 0.06	0.734 ± 0.06
PO$_2$ (mmHg)	74 ± 5.3	77 ± 4.7
PCO$_2$ (mmHg)	28.6 ± 2.1	27.4 ± 1.9
pH	7.46 ± 0.018	7.46 ± 0.018
Organ	*Flows (ml/min)*	
Carcass	110.4 ± 13.7	108.6 ± 12.6
Skin	22.5 ± 4.0	20.2 ± 4.0
Right hind leg	12.7 ± 2.2	11.3 ± 2.0
Left hind leg	11.3 ± 2.0	10.9 ± 2.1
Intestines	64.0 ± 10.1	56.2 ± 10.2
Stomach	15.2 ± 2.5	9.5 ± 1.9*
Liver	14.0 ± 2.4	14.1 ± 2.7
Right kidney	46.8 ± 5.3	43.2 ± 4.5
Left kidney	46.3 ± 5.4	43.0 ± 4.4
Heart	14.6 ± 1.7	15.0 ± 1.5
Brain	3.1 ± 0.28	3.5 ± 0.17

* $P < 0.002$.

Hydralazine

Nine rabbits of 2.6 ± 0.12 kg body weight were injected with microspheres before, and 20-30 min after, administration of hydralazine 200 μg/kg intravenously (Table 2). There was a large fall in arterial pressue to 45 mmHg (by 46%). Total peripheral resistance was reduced by 55% on average, cardiac output increased by 20% and heart rate by 11%. The change in stroke volume was insignificant.

The vascular resistances of the carcass, heart, brain ($P < 0.001$), legs, kidneys ($P < 0.01$) and skin ($P < 0.05$) fell significantly. It is interesting to note that calculated skin vascular resistance fell in spite of the reduction in skin blood flow. It is remarkable that in spite of the large reduction in arterial pressure there was no evidence of compensatory vasoconstriction in the organs selected for flow measurement.

Table 2 also shows that administration of hydralazine in the dose used caused an increase in ventilation which was noticeable at the time and is shown by the rise of $Pa\mathrm{O}_2$ and fall in $Pa\mathrm{CO}_2$. This is probably due to peripheral arterial chemoreceptor stimulation consequent on the fall in arterial pressure. Once again the absence of concomitant vasoconstriction in the peripheral circulation (so far as this was analysed) is interesting. The evidence suggests that hydralazine had caused very widespread, overriding and non-selective vasodilatation.

SK&F 24260

Fourteen rabbits of 2.8 ± 0.12 kg were injected with microspheres before and 20-30 min after administration of SK&F 24260 200 μg/kg subcutaneously. The mean arterial pressure fell to 52 mmHg (by 38%, Table 3). Total peripheral resistance was reduced by 39%. There was no significant change in heart rate, cardiac output or blood gas values.

Among the changes in the distribution of cardiac output was a two-fold rise in cerebral flow, due to a 72% fall in cerebral vascular resistance. The vascular resistances of the carcass, legs, brain, large intestines ($P < 0.001$) and heart ($P < 0.05$) also were reduced. Those of other organs were not altered significantly.

Guanethidine

Eleven rabbits, of 2.69 ± 0.18 kg, were injected with microspheres before and 20-30 min after administration of guanethidine 500 μg/kg intramuscularly. The mean arterial pressure fell to 75 mmHg (by 23%, Table 4). Total peripheral resistance was reduced by 15%. There was a small increase in heart rate; the mean reduction in cardiac output was not significant but there was a significant decrease in stroke volume ($P < 0.01$). Blood gas values did not change significantly.

As Table 4 shows, administration of guanethidine caused a widespread reduction in the

Table 2 Effects of hydralazine (200 μg/kg, i.v.) in nine rabbits

	Initial	*After drug*	P<
Cardiac output (ml kg^{-1} min^{-1})	119.4 ± 5.6	143.4 ± 10.5	0.01
Arterial pressure (mmHg)	84.3 ± 4.2	45.1 ± 3.8	0.001
Heart rate (beats/min)	308 ± 14.8	343 ± 21.1	0.01
Total peripheral resistance	0.76 ± 0.04	0.34 ± 0.02	0.001
$P\mathrm{O}_2$ (mmHg)	70 ± 2.5	84 ± 3.1	0.01
$P\mathrm{CO}_2$ (mmHg)	29 ± 1.1	26 ± 1.2	0.05
pH	7.45 ± 0.02	7.48 ± 0.01	NS
Organ	*Flows (ml/min)*		
Carcass	89.0 ± 10.1	103.5 ± 12.6	0.05
Skin	21.8 ± 3.1	13.6 ± 2.4	0.01
Right hind leg	10.6 ± 1.9	9.8 ± 2.5	NS
Left hind leg	10.3 ± 1.8	8.5 ± 2.0	NS
Intestines	49.5 ± 4.2	54.0 ± 9.9	NS
Stomach	9.4 ± 1.0	4.2 ± 0.6	0.002
Liver	15.2 ± 3.2	14.1 ± 2.3	NS
Right kidney	35.6 ± 2.8	53.0 ± 5.8	0.002
Left kidney	35.1 ± 2.9	55.8 ± 4.6	0.001
Heart	12.1 ± 1.9	31.1 ± 6.7	0.01
Brain	3.6 ± 0.5	3.8 ± 0.5	NS

NS = Not significant.

blood flow to organs and tissues. None of the changes in vascular resistances was statistically significant, except that of the thyroid, which was increased more than three-fold.

The results of the four sets of experiments are shown in Figure 1. It is evident that administration of the three drugs caused much larger changes in arterial pressure and in the redistribution of

Table 3 Effects of SKF 24260 (200 μg/kg, s.c.) in 14 rabbits

	Initial	*After drug*	P<
Cardiac output (ml kg^{-1} min^{-1}	154.0 ± 5.7	153.3 ± 10.7	NS
Arterial pressure (mmHg)	84.1 ± 3.8	52.3 ± 4.1	0.001
Heart rate (beats/min)	321 ± 11.8	320 ± 11.8	NS
Total peripheral resistance	0.596 ± 0.031	0.364 ± 0.4	0.001
P_{O_2} (mmHg)	71 ± 2.8	69 ± 3.0	NS
P_{CO} (mmHg)	29 ± 1.6	28 ± 2.2	NS
pH	7.46 ± 0.009	7.41 ± 0.023	NS
Organ	*Flows (ml/min)*		
Carcass	140.0 ± 12.1	160.4 ± 16.0	NS
Skin	25.9 ± 3.3	18.6 ± 3.0	NS
Right hind leg	14.4 ± 0.87	17.6 ± 2.2	NS
Left hind leg	13.8 ± 0.75	17.0 ± 2.1	NS
Small intestine	38.8 ± 4.2	28.5 ± 3.1	0.05
Large intestine	30.0 ± 2.5	32.1 ± 3.6	NS
Stomach	16.6 ± 1.98	12.2 ± 1.6	NS
Liver	20.2 ± 3.4	20.6 ± 2.7	NS
Right kidney	51.6 ± 3.8	35.4 ± 4.2	0.002
Left kidney	50.7 ± 3.6	35.6 ± 4.2	0.002
Heart	17.9 ± 1.8	25.1 ± 3.1	0.05
Brain	3.9 ± 0.47	8.7 ± 1.8	0.02
Spleen	4.06 ± 1.2	1.9 ± 0.44	0.05

Table 4 Effects of guanethidine (500 μg/kg, i.v.) in 11 rabbits

		Initial	*After drug*	P<
Cardiac output (ml kg^{-1} min^{-1})		167.7 ± 12.4	144.9 ± 8.9	NS
Arterial pressure (mmHg)		97.0 ± 2.9	74.6 ± 3.7	0.001
Heart rate (beats/min)		340 ± 13.9	370 ± 13.9	0.001
Total peripheral resistance		0.612 ± 0.04	0.522 ± 0.02	0.02
P_{O_2} (mmHg)		75 ± 5.2	78 ± 4.1	NS
P_{CO_2} (mmHg)		28.2 ± 2.1	25.6 ± 1.8	NS
pH		7.46 ± 0.19	7.43 ± 0.02	NS
Organ	*Weights (g)*	*Flows (ml/min)*		
Carcass	1275 ± 12	118.1 ± 5.8	109 ± 9.5	NS
Skin	391 ± 36	31.9 ± 2.4	20.3 ± 2.6	0.002
Right hind leg	208 ± 14	16.5 ± 1.1	13.2 ± 1.0	0.05
Left hind leg	203 ± 13	16.2 ± 1.1	13.2 ± 1.0	0.05
Small intestine	108 ± 6	45.6 ± 4.1	37.2 ± 5.1	0.05
Large intestine	201 ± 9	34.1 ± 2.9	32.1 ± 3.3	NS
Stomach	82 ± 11	16.1 ± 3.0	11.1 ± 2.0	0.05
Liver	119 ± 7	13.6 ± 1.2	17.3 ± 3.5	NS
Right kidney	10.4 ± 0.6	42.9 ± 7.0	33.7 ± 6.4	0.05
Left kidney	10.5 ± 0.6	43.2 ± 6.9	33.0 ± 6.4	0.05
Heart	7.5 ± 0.4	17.5 ± 1.7	16.8 ± 1.7	NS
Brain	7.8 ± 0.3	4.0 ± 0.43	3.72 ± 0.2	NS
Spleen	1.06 ± 0.08	3.3 ± 0.56	2.8 ± 0.86	NS
Thyroid	0.192 ± 0.02	0.225 ± 0.07	0.062 ± 0.017	0.02
Adrenals	0.235 ± 0.03	0.230 ± 0.045	0.319 ± 0.06	0.05

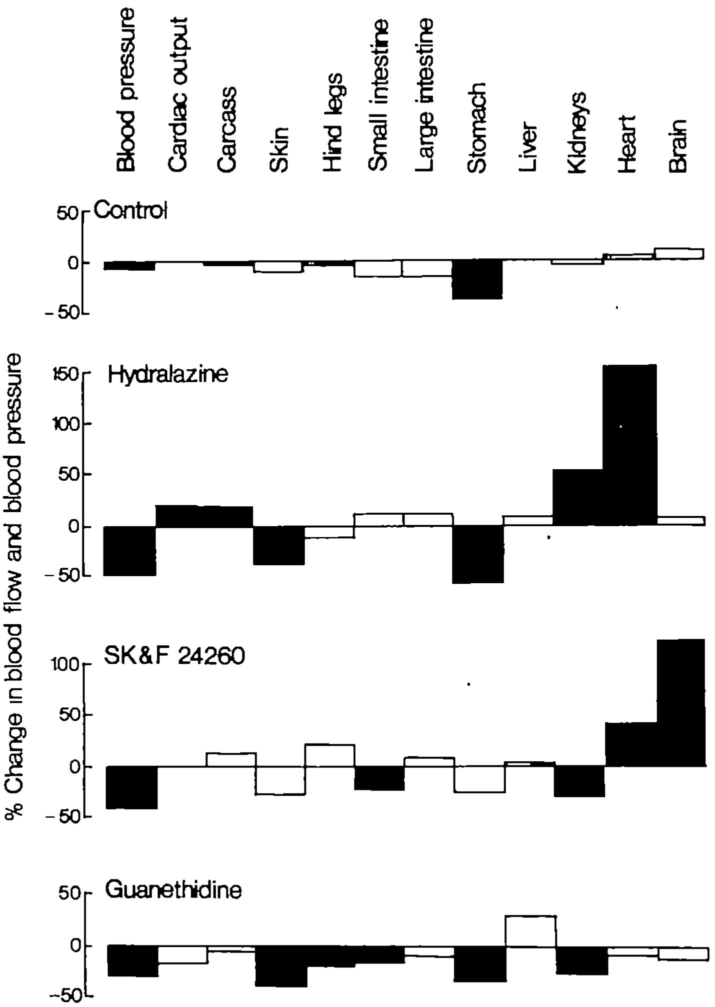

Figure 1 Percentage change from resting blood flow to all organs 30 min after hydralazine, guanethidine and SK&F 24260 compared with the fall in blood pressure. Note: (1) Shaded areas are those in which the changes in blood flow were statistically significant. (2) The controls show the change in flow and pressure between two microsphere injections 30 min apart.

cardiac output than occurred spontaneously in the group of rabbits which received no drug. The changes in arterial pressure in the three experimental groups were different, and the consequential homeostatic physiological responses (through activation of the baroreceptor and chemoreceptor reflexes and of the renin-angiotensin system) may well have differed. Nevertheless the doses were selected on the basis of those used clinically, and from this point of view, but bearing in mind species differences, are comparable.

With these reservations the results show a remarkable difference in the cardiovascular responses to hydralazine and SK&F 24260 (Figure 1), especially as regards the coronary, cerebral and renal blood flows. Comparison between the effects of these two drugs and that of guanethidine is even more striking.

Discussion

The results show that the radioactive microsphere method for measuring cardiac output and its

Table 5 Percentage distribution of cardiac output in adult rabbits

	Chalmers et al. *(1967a)*	*Neutze* et al. *(1968)*		*Warren (1973)*		*Present Paper*
	Local thermodilution	*Microspheres*		*Microspheres*		*Microspheres*
	Unanaesthetized	*Unanaesthetized*	*Halothane*	*Unanaesthetized*	*Pentobarbitone*	*Pentobarbitone*
Total body muscle	33.6					
Carcass		39 6*	36.5*	29.0	39.6	29.2
Limbs				12 9	15 1	6.7†
Skin	11.3	7.4	5.8	5.0	7.1	6.5
Small intestine		11.5	11.1	7.3	5.6	10 0
Large intestine	16.6‡	9.3	11.6	7.8	5.9	8.1
Stomach		3.4	4.3	3.9	1.9	3.5
Kidneys	20.0	16.2	16.6	14.7	12.0	21.0
Heart		2.8	3.7	2.9	2.1	4.1
Brain		1.2	1.8	0.9	0.5	0.9
Spleen		1.8	1.9	1.1	0.3	0.8
Liver		3.4	5.4	2.9	2.9	3.8

* Including hind limbs. † Hind limbs only. ‡ Portal venous.

distribution is useful for detecting changes simultaneously in many organs, and that different drugs which lower arterial pressure cause very different cardiovascular changes. Figure 1 shows that the reduction in blood pressure varied with the different drugs and while this may influence the cardiovascular changes that were seen, the aim of the experiment was to select drug dose on the basis of those used clinically.

The cardiac output in the present experiments on rabbits under light pentobarbitone anaesthesia (145 ± 4.9 ml kg^{-1}min^{-1}) was less than in unanaesthetized rabbits (175-250 ml kg^{-1}min^{-1}) as estimated by direct Fick or indicator dilution methods (Korner & Darian Smith, 1954, Edwards, Korner & Thorburn, 1959, Korner, 1963, 1965; Chalmers, Korner & White, 1967a, b, c; Warren, 1973) but greater than in some previously reported series under pentobarbitone (e.g. 110 ml min^{-1}kg^{-1}.; Neutze, Wyler & Rudolph, 1968). Within the present series there were large variations in the mean initial cardiac outputs (119 ± 6 ml min^{-1}kg^{-1} in those which subsequently received hydralazine; 168 ± 12 ml min^{-1}kg^{-1} in those which later were given guanethidine) for no apparent reason (size or source of rabbits, thermal, anaesthetic dose, duration of experiment, season). Nevertheless the proportionate distribution of cardiac output to different organs and tissues did not vary significantly. The distribution was very similar to that observed by others in unanaesthetized or anaesthetized rabbits (Table 5), although Warren (1973) found a greater proportion of cardiac output distributed to skeletal muscle and less to the viscera. This is probably because his rabbits were larger; the carcass carried a greater proportion of the extra weight than the abdominal organs.

The effect of hydralazine on cardiac output and on the blood flow to many organs is well documented in both animals and man. The increase in cardiac output observed in rabbits using microspheres agrees with that in clinical studies by Wilkinson, Backman & Hecht (1952), Fries, Rose, Higgins, Finnerty, Kelley & Partenope (1953) and Stein & Hecht (1955). Similarly the large increases in renal and coronary flows agree with those found in man by Reubi (1950), Wilkinson *et al.* (1952), Stein & Hecht (1955) and Crumpton, Rowe, Crosley, Maxwell & Huston (1953). Hydralazine is believed to lower blood pressure by dilatation of the pre-capillary resistance vessels (Ablad, 1963). Although the vascular resistance fell in the heart and brain, even if these organs were maximally dilated they would not contribute greatly to the fall observed in total peripheral resistance; in rabbits the heart normally only receives about 4-5% and the brain 1% of cardiac output. The simplest way of expressing the contribution of the vascular beds of different organs and tissues to the total, is to consider it in terms of vascular conductances. Table 6 shows the changes in total systemic vascular conductances on administration of hydralazine and SK&F 24260. It also shows the relative contributions of the principal organs to the mean change, where this contribution is equal to, or exceeds 10% of the total change. For hydralazine the greatest effects are in the carcass and kidneys.

Like hydralazine, SK&F 24260 has been described as a pre-capillary vasodilator (Fielden, Owen & Taylor, 1974). The present observations show that it causes a large fall in blood pressure, due to a decrease in total peripheral resistance with no change in cardiac output. Although the increases in blood flow to the carcass and hind legs were not statistically significant, increases in vascular conductance were significant and large (Table 6). Together they contributed to nearly 63% of the observed increase in total peripheral conductance. As with hydralazine, SK&F 24260 caused a significant increase in vascular conductance to the heart. Similarly, SK&F 24260

Table 6 Changes in systemic vascular conductance in rabbits given hydralazine or SKF 24260

	Hydralazine	*SKF 24260*
Total systemic conductance	1.32	1.68
Change	+ 1.63 (123%)	+ 1.07 (63%)
% of total change* in.		
Carcass	+31	+48
Hind limbs		+12
Intestine	+15	+10
Kidneys	+38	
Heart	+13	+10

* Only those changes which were statistically significant, and which, for different organs, accounted for 10% or more of the total.

also caused a significant increase in vascular conductance in the large intestines. Yet the increases in conductance in the heart and intestine each contribute only 10% of the total, so the major site of action of SK&F 24260 is in skeletal muscle. The very large increase in cerebral flow produced by SK&F 24260 is interesting and seems worth further investigation, although it contributes very little to the overall lowering of blood pressure.

The cardiovascular changes produced by guanethidine were very different from those caused by hydralazine or SK&F 24260. Guanethidine is thought to have a selective action on the post-ganglionic nerves (Maxwell, Mull & Plummer, 1959; Maxwell, Plummer, Schneider, Povalski & Daniel, 1960) by interfering with the synthesis of noradrenaline (Abercrombie & Davies, 1963) or by otherwise depleting the local stores. Fielden & Green (1967) concluded that guanethidine also had a strong adrenergic neurone blocking action distinct from the depleting action. It has been suggested that a reduction in total peripheral resistance (by blocking the sympathetic nervous system) is the mechanism by which guanethidine lowers blood pressure (Chamberlain & Howard, 1964). In the present experiments the total peripheral conductance was only increased by 17% after guanethidine; there was no significant change in any individual organ. Guanethidine caused a significant fall in stroke volume, and it is likely that this is the principal means by which blood pressure was reduced. Sympathetic vasoconstrictor nerves are distributed not only to the arterioles but also to the venules and veins. Therefore a decrease in sympathetic tone would increase the venous capacity of the peripheral vascular beds and so reduce venous return.

Guanethidine is very different from hydralazine and SK&F 24260 because it did not cause vasodilatation in any vascular bed. The widespread fall in systemic blood flow after guanethidine (Table 4) is small and must be a passive result of the lowered blood pressure, because the calculated vascular conductances to all organs were not changed significantly in the conditions of these experiments.

In the group of rabbits which received guanethidine it was also decided to look at the effects of the drug on certain endocrine glands, namely the thyroid and the adrenal. As Table 4 shows they reacted very differently from the other organs; the blood flow to the thyroid was much reduced, the resistance being significantly increased. The adrenal glands were the only organs to show a significant increase in flow. These results were unexpected and deserve further investigation.

I wish to thank Dr N.W. Bruce for advice and encouragement and Professor G.S. Dawes for valuable criticism and help in the preparation of this manuscript. This work was supported by a grant from Smith Kline & French Limited

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(Received May 8, 1975.
Revised July 15, 1975.)

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ACTIONS OF PROSTAGLANDIN E_2 ON MYOCARDIAL MECHANICS, CORONARY VASCULAR RESISTANCE AND OXYGEN CONSUMPTION IN THE GUINEA-PIG ISOLATED HEART PREPARATION

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1 In isolated, electrically driven (3 Hz) hearts of guinea-pigs the action of prostaglandin E_2 on left ventricular pressure (LVP), oxygen consumption (QO_2) and coronary vascular resistance (CVR) was studied by establishing cumulative concentration-response curves. The hearts were perfused at a constant flow (10 ml/min) with Tyrode solution (Ca^{++} 1.8 mM) at 32° C.

2 Under control conditions prostaglandin E_2 (2.86 x 10^{-11} – 1.43 x 10^{-7}M) decreased LVP, QO_2 and CVR in a concentration-dependent manner by maximally 27, 18 and 38%, respectively ($P < 0.05$).

3 After reserpine pretreatment there were lower initial values for all parameters measured. The effect of prostaglandin E_2 on LVP and QO_2 was abolished, but CVR was further diminished, depending on the concentration.

4 The results seem to support the hypothesis of an interaction of prostaglandin E_2 with endogenous catecholamines as far as the effects on LVP and QO_2 are concerned. In contrast, prostaglandin E_2 seems to have a direct action on CVR, which is independent of the presence of catecholamines.

Introduction

Investigations into the action of prostaglandins on myocardial muscle preparations have been the subject of numerous studies (for references see Nakano, 1973). However, the results were divergent and generally acceptable explanations for these differences (which obviously seem to be more than simple species differences) are lacking. One possible cause may be the different methods of investigation. Thus, it is difficult to draw definite conclusions about changes in myocardial contractile force by prostaglandins from experiments with spontaneously beating preparations, since it is well known that the prostaglandins of the E series in particular also possess marked chronotropic actions (for references see Hedquist, 1973). Furthermore, especially for the E prostaglandins, their possible interaction with adrenergic neurotransmission must be taken into account; this in itself should induce alterations in myocardial contractile force and heart rate (see Hedquist, 1973).

Finally, there are striking differences between the atrial and ventricular myocardium, in their morphology (Forssmann & Girardier, 1966; Fawcett & McNutt, 1969; McNutt & Fawcett, 1969) as well as in their responsiveness to stimulation by exogenous drugs (Webler, 1974). These seem to have been overlooked at least by those authors, who tend to extend their results, obtained in isolated atrial preparations to the heart in general.

It was the aim of this study to investigate the actions of prostaglandin E_2 on factors, which are assumed to be of importance for myocardial performance, such as left ventricular pressure development, myocardial oxygen consumption and coronary vascular resistance in well defined isolated ventricle preparations in relation to their basic adrenergic activity.

Methods

Design of the experiment

In guinea-pigs (300-400 g body weight) of either sex, thoracotomy was performed under light ether anaesthesia after pretreatment with heparin (10 mg/kg i.p.). The aorta was cannulated as soon as possible and the heart perfused according to the

Langendorff technique with Tyrode solution at room temperature during the time of preparation, including cannulation of the pulmonary artery and ligation of all other blood vessels near the heart. A fluid-filled rubber-balloon-catheter was inserted into the left ventricle via the mitral ostium and connected with a Statham P 23 Db pressure transducer for measuring left ventricular actively developed pressure (LVP) and its derivatives: contraction velocity (LVdP/dt_{max}) and relaxation velocity (LVdP/dt_{min}). Characteristics of the system for pressure measurement were: resonant frequency 100 Hz, quenching factor about 0.5, time constant 1 ms, maximal frequency response. 24 Hz. Time to peak tension (TPT), which is not influenced by changes in pressure development, was estimated graphically from recordings at high paper speed (100 mm/second). Myocardial oxygen consumption (QO_2) was monitored by polarimetry (platinum-electrodes, Clark principle, sensitivity: 25-33 nA/mmHgO_2, provision for adjustment: 90% of final value within <1 s) of the pulmonary artery outflow as described elsewhere (Klaus & Krebs, 1969). In order to prevent limitation in oxygen supply to the heart in each experiment oxygen tension (PO_2) was about 650 mmHg in the inflowing solution and higher than 200 mmHg in the outflow.

During the experiments the heart was perfused at constant flow (10 ml/min) with Tyrode solution of the following composition (mM); Na^+ 149, K^+5.4, Ca^{2+}1.8, Mg^{2+} 1.05, Cl^- 145, $H_2PO_4^-$0 42, HCO_3^-11.9, THAM 10.0, disodium edetate (EDTA) 0.05, glucose 11.0, pH 7.4, equilibrated with 95% O_2 and 5% CO_2 at 32° C and electrically driven at a constant rate of 180 beats/min (Grass stimulator S5, impulse duration 4 ms, 40V).

Coronary perfusion pressure was measured by Statham P 23 Bb pressure-transducer in the coronary inflow tract and was considered to be a direct expression of coronary vascular resistance (CVR), because the perfusion flow was held constant.

After an equilibration period of about 20 min, prostaglandin E_2 was added in a cumulative way. Experiments did not last longer than 120 minutes. In some animals reserpine (Serpasil) 7 mg/kg was given intraperitoneally once 16-24 h before the experiment.

Preparation of prostaglandin E_2 solutions

Stock solutions of prostaglandin E_2 were prepared as follows: 5 mg was dissolved in 0.5 ml 95% ethanol, 4.5 ml Na_2CO_3 (20 mg/100 ml) was added, the final pH checked (6-7.5), and the solution was stored frozen at −25° C not longer than 1 month. Immediately before starting an experiment dilutions were made from the stock solutions to give a concentration of 5 μg/ml prostaglandin E_2, aliquots of which were added to the perfusion fluid.

Statistical analysis

Statistical evaluation was performed using Student's *t*-test for comparing the means of the variables, which were tested with and without maximum concentrations of prostaglandin E_2. The coefficient of regression (b_{yx}) as obtained by analysis of covariance was also tested for significance. The means and their standard error ($\bar{x}$ ± s.e. mean) are quoted in the text. *n* is the number of observations. The level of significance for α in each case was 0.05.

Results

Time of constant performance, coronary vascular resistance and O_2 consumption

In a first series of experiments LVP (mmHg), left ventricular contraction (LVP/dt_{max}) and relaxation (LVdP/dt_{min}) velocities (mmHg/s), time to peak tension (TPT) (ms), mean CVR (mmHg/ml) and QO_2 (μl O_2/g ventricular dry weight per min) were measured under control conditions over a total time of 120 min after stabilization of the preparations from reserpine-treated and non-treated animals. Apart from a small and insignificant decrease of about 4.5% in QO_2 in non-treated animals essentially no changes in any of the parameters were observed ($n = 6$). Thus, at least for a period of 120 min, our experimental conditions may be assumed to maintain a stable performance of the heart preparations.

Influence of reserpine-treatment

In the experiments on the possible role of catecholamines in the action of prostaglandin E_2, hearts from reserpine-treated animals were used. The actual degree of catecholamine depletion was checked by the addition of tyramine (5 μg/ml) to the perfusion fluid, in another series of experiments. Tyramine produced only small increases in myocardial performance without alterations in CVR and QO_2, indicating a sufficient depletion of catecholamines by reserpine.

As summarized in Table 1, there was a marked diminution by reserpine treatment of myocardial contractile force and CVR. On the other hand, only an insignificant decrease in QO_2 was observed.

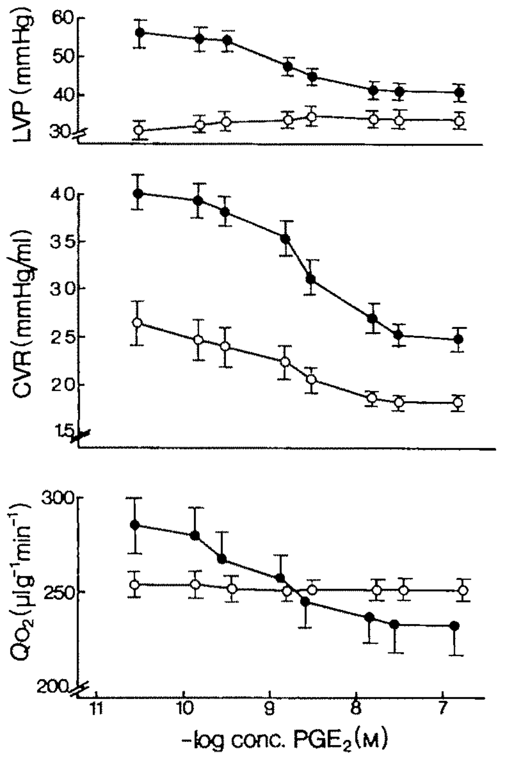

Figure 1 Changes in left ventricular pressure (LVP) (mmHg), coronary vascular resistance (CVR) (mmHg/ml) and oxygen consumption QO_2 (µl/g ventricular dry weight per min.) in response to – log molar concentration of prostaglandin E$_2$ (PGE$_2$) in control ((●) $n = 8$) and reserpine pretreated ((○) ,$n = 8$) animals. Vertical lines show s.e. mean.

Effects of prostaglandin E_2 in control animals

Prostaglandin E$_2$ in concentrations ranging from 2.86×10^{-11} to 1.43×10^{-7}M was studied. At 2.86×10^{-11}M prostaglandin E$_2$ did not alter myocardial performance, CVR or QO_2. However, if the concentration of prostaglandin E$_2$ was raised, there occurred a concentration-dependent decrease in cardiac performance, coronary vascular resistance and oxygen consumption. Changes in LVP, CVR, and QO_2 are shown in Figure 1 (●), changes in LVdP/dt_{max}, LVdP/dt_{min} and TPT in Table 2 (left side). All changes, except the decrease in QO_2 became statistically significant at 2.86×10^{-9}M prostaglandin E$_2$, if compared with the respective control values. However, for QO_2 a significant regression-line with $b_{yx} = -21.63 \pm 3.11$ was obtained.

Effects of prostaglandin E_2 in reserpine-treated animals

In reserpine-treated guinea-pigs the effects of the same range of prostaglandin E$_2$ concentrations, i.e. 2.86×10^{-11} to 1.43×10^{-7}M, were studied. In contrast to control animals, in reserpine-treated guinea-pigs which had lower control levels of LVP, CVR and QO_2, there were no further changes in LVP and QO_2, when additive concentrations of prostaglandin E$_2$ were applied (Figure 1 (○)). The same was true for LVdP/dt_{max}, LVdP/dt_{min} and TPT (Table 2, right side).

In contrast, a concentration-dependent decrease in CVR was also seen after the animals had been reserpine-treated: the difference became significant at 2.86×10^{-9}M prostaglandin E$_2$, i.e. at the same concentration as in the non-treated group. However, the coefficient of regression was only -0.38 ± 0.04 as compared with -0.72 ± 0.04 ($P < 0.05$) in the non-reserpine-treated group. Thus, the curves are not parallel. Changes in LVP and CVP are demonstrated in representative experiments with a control heart and a heart from a reserpine-treated guinea-pig in Figure 2.

Discussion

The above results provide clear evidence for a concentration-dependent decrease in myocardial performance, coronary vascular resistance and myocardial oxygen consumption in constant-flow perfused, electrically stimulated isolated heart of

Table 1 Left ventricular pressure (LVP), left ventricular contraction velocity (LVdP/dt_{max}) and relaxation velocity (LVdP/dt_{min}), time to peak tension (TPT), mean coronary vascular resistance (mCVR) and oxygen consumption (QO_2) of control and reserpine-treated guinea-pigs

	n	*LVP* *(mmHg)*	*LVdP/dt$_{max}$* *(mmHg/s)*	*LVdP/dt$_{min}$* *(mmHg/s)*	*TPT* *(ms)*	*mCVR* *(mmHg/ml)*	QO_2 *(µlg^{-1}min^{-1})*
Control	8	55.8 ± 3.6	742 ± 53	501 ± 41	117.5 ± 2.5	4.00 ± 0.18	285 ± 15
Reserpine	8	30.4 ± 2.1	374 ± 28	249 ± 18	136.9 ± 1.6	2.69 ± 0.25	254 ± 13
Significance		+	+	+	+	+	NS

QO_2 = µl O$_2$ per g ventricular dryweight and per minute.

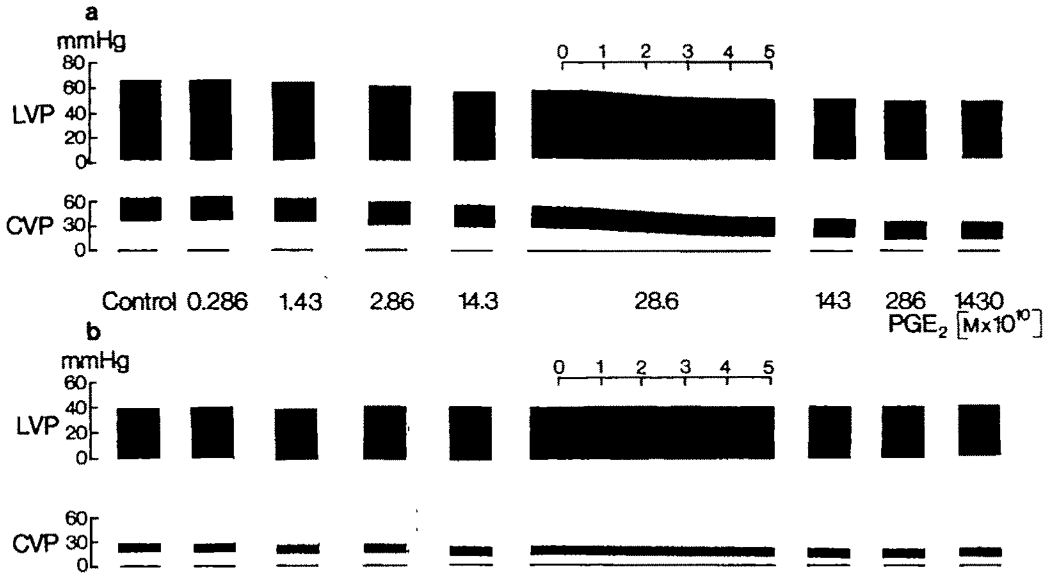

Figure 2 Changes in left ventricular pressure (LVP) and coronary vascular pressure (CVP) produced by increasing concentrations of prostaglandin E_2 (PGE_2) in representative control (a) and reserpine-pretreated animals (b). At 2.86×10^{-9}M, a continuous record lasting 5 min is shown; at the other concentrations of prostaglandin E_2 only the final steady effect is shown. Note the differences in initial values of LVP and CVP between (a) and (b), the decrease in LVP for (a) but not (b) and the diminution of CVP for both groups, although more pronounced for (a), with the same final value.

Table 2 Changes in left ventricular contraction velocity ($LVdP/dt_{max}$), relaxation velocity ($LVdP/dt_{min}$) and time to peak tension (TPT) by cumulative addition of prostaglandin E_2 (PGE_2) in reserpine-treated and non-treated animals

	No reserpine (n = *8*)			*Reserpine* (n = *8*)		
PGE_2 (M)	*$LVdP/dt_{max}$ (mmHg/s)*	*$LVdP/dt_{min}$ (mmHg/s)*	*TPT (ms)*	*$LVdP/dt_{max}$ (mmHg/s)*	*$LVdP/dt_{min}$ (mmHg/s)*	*TPT (ms)*
2.86×10^{-11}	742 ± 53	509 ± 41	118 ± 2	381 ± 27	262 ± 19	137 ± 2
1.43×10^{-10}	727 ± 49	489 ± 33	120 ± 2	401 ± 25	268 ± 20	134 ± 2
2.86×10^{-10}	684 ± 38	468 ± 23	120 ± 2	404 ± 23	266 ± 19	134 ± 2
1.43×10^{-9}	618 ± 39	424 ± 23	125 ± 3	429 ± 25	274 ± 18	133 ± 2
2.86×10^{-9}	571 ± 36*	401 ± 21*	126 ± 3*	432 ± 25	282 ± 21	132 ± 2
1.43×10^{-8}	528 ± 37*	368 ± 20*	129 ± 3*	430 ± 24	279 ± 18	132 ± 2
2.86×10^{-8}	525 ± 39*	366 ± 19*	129 ± 3*	437 ± 25	282 ± 18	132 ± 2
1.43×10^{-7}	521 ± 37*	364 ± 17*	129 ± 3*	437 ± 25	284 ± 19	132 ± 2

Values are mean ± s.e. mean

* Significant in comparison with value in presence of 2.86×10^{-11}M prostaglandin E_2

the guinea-pig by prostaglandin E_2. This is in contrast to many investigations on the actions of E-prostaglandins, carried out with isolated atria or spontaneously beating heart preparations, where either no changes or increases in contractile force were described (for references see Nakano, 1973).

On the other hand, our results agree well with an investigation reported by George, Paddock & Kadowitz (1972), who described a marked decrease in contractile force of isolated, electrically driven hearts of rats by prostaglandin E_1 in a concentration of 1×10^{-8}M. However, the transient preceding increase in contractile force as well as a tendency for contractile force to return towards control values, while the perfusion was continued (George *et al*, 1972), could not be detected under our conditions. As shown in this paper, the effect of prostaglandin E_2 was essentially stable, which was confirmed in a more detailed study, that gave no evidence for tachyphylaxis to prostaglandin E_2 in either guinea-pig ventricles or atria (Krebs & Schrör, 1975).

A probable explanation of the decrease in contractile force could be seen in the fact that the electrical stimulation of cardiac sympathetic fibres, which are involved in the maintenance of constant myocardial performance in non-treated animals, is inhibited by prostaglandin E_2. This is in agreement with previous reports, in which inhibition by prostaglandin E_1 and E_2 of adrenergic transmitter release in the sympathetically innervated rabbit heart (Hedquist & Wennmalm, 1971) and decrease by prostaglandin E_1 of the release of noradrenaline in the rat heart *in situ* with an intact nervous system (Papanicolaou, Meyer & Milliez, 1971) were found. This hypothesis is further supported by the lack of any effects on myocardial performance, if the animals are reserpine-treated, i.e. depleted of their endogenous catecholamine content.

On the other hand, there is good evidence in favour of a direct dilatory action of prostaglandin E_2 on coronary vessels, because this effect persisted also in catecholamine-depleted hearts. However, the participation of some residual catecholamines, combined with a greater responsiveness of coronary vessels than of ventricular myocardium to catecholamine action offers another possible explanation.

Hence, if effects of prostaglandins in isolated hearts are studied, the endogenous transmitter content has to be considered in every case. This seems to have a greater influence in ventricular than in left atrial preparations of the same animal. In contrast to ventricular muscle, the contractile force of atrial muscle was increased as a result of a direct action of prostaglandin E_2. This effect on atrial muscle could even be enhanced by prior reserpine-treatment (Krebs & Schror, 1975). The result, that the isoprenaline concentration-response curve for increase in actively developed left ventricular pressure is shifted to the right and downwards in control but not in reserpine-treated animals (Ca^{++} 1.8 mM) (Schrör, Becker, Berg & Krebs, 1975), provides additional evidence for the view that many of the differences reported, e.g. so-called 'inotropic' actions, seem to be understandable on the basis of the different models and methods of investigations used.

The work was supported by the Deutsche Forschungsgemeinschaft. The authors wish to thank Dr F. Brunnberg, The Upjohn Company, for generously supplying prostaglandin E_2.

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Br. J. Pharmac. (1975), 55, 409-413

VOLUNTARY LITHIUM INTAKE, 'ANTIDOTAL THIRST' AND CONCURRENT BEHAVIOUR OF RATS

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1 Voluntary intake of various pair combinations of fluids (100 mM, 10 mM LiCl, 10 mM NaCl, water) and body weight was measured daily in rats.

2 More lithium was consumed when water was available.

3 When offered a lithium-sodium choice the rats did not consume significantly more saline than water on the previous trial. While saline consumption increased over the two days, lithium decreased slightly.

4 Following the lithium-only trial, water and saline were provided. Marked polydipsia was observed on the first day and the rats drank more water than saline. On the second day there was a significant drop in saline intake while water consumption returned to baseline levels.

5 Behavioural measurements overall confirmed the depressant effect of lithium: decreased ambulation and rearing and increased time spent immobile/grooming.

6 These findings are discussed with reference to lithium toxicity, which may be a confounding variable in studies concerned with the effects of this salt on the behaviour of laboratory rodents. Behavioural irritability such as aggression reported in situations using long-term lithium treatment may be reduced by provision for voluntary saline consumption.

Introduction

Voluntary ingestion of lithium and sodium salts by laboratory rats has been the subject of two separate areas of investigation. Firstly, the ability of these animals to discriminate between chloride solutions has provided a useful paradigm for neuro-physiological (e.g., Beidler, 1953) and learning (e.g., Strom, Lingenfelter & Brody, 1970) theorists. Secondly, the use of lithium as a prophylactic for manic-depressive states has prompted examination of electrolyte metabolism, since sodium levels may influence the renal excretion of lithium and thus its therapeutic efficacy (Thomsen, 1973).

One side effect of prolonged treatment with lithium salts in rats is the development of polydipsia and polyuria. The causal sequence of these symptoms, however, is unclear although their similarity to those of diabetes insipidus has been attributed to the reversible inhibition of anti-diuretic hormone in the rat kidney (Harris & Jenner, 1968).

Smith, Balagura & Lubran (1970) suggest that rats exhibit 'antidotal thirst' for water which acts, through the renal system, to rid the body of toxic lithium ions. Evidence has since emerged to show that high doses of lithium can impair conservation of sodium, so that rats administered lithium in food choose to drink saline rather than water, thus minimizing lithium toxicity (Schreiber & Roháčová, 1971; Thomsen, Jensen & Olesen, 1974). Furthermore, an inverse relationship between the intake of lithium and of sodium chloride solutions has been noted (Strom *et al.*, 1970; Ellman & Gan, 1973).

The present work examines the voluntary consumption of the two salts (LiCl, NaCl) and water by rats, using a two-bottle choice situation and baseline water intake measures. This procedure differs from those used in earlier studies of lithium toxicity in that administration of the salt is neither masked in food (e.g., Thomsen *et al.*, 1974) nor introduced directly to the stomach (e.g., Smith *et al.*, 1970). Also, no previous designs have incorporated a water/saline choice following short-term voluntary intake of small amounts of lithium in the drinking water.

Since lithium has been reported to depress the activity of laboratory rats (Johnson & Wormington, 1972; Smith & Smith, 1973; Syme & Syme, 1973, 1974) the non-directed behaviour of individual subjects was observed daily in an open field to determine any gross effects of the experimental manipulations.

Methods

Male hooded rats (67-168 g) were housed singly and measures of water intake and body weight were obtained for 10 days. Each cage measured 25 x 27 x 13 cm and two water bottles were situated 3.5 cm from the edge and in the middle of the front wall. Standard laboratory food was freely available in hoppers attached to the side wall. Details of solutions presented, including the 10-day baseline water measures, are shown in Table 1. Pairs were switched on alternate days to avoid position preferences for particular bottles. The solutions were diluted to 10 mM after the first trial (days 11-12) since the experimental error involved in volume measurements (± 0.5 ml) precluded accurate assessment of the amount consumed, this being about 1 ml of 0.1 M LiCl solution.

Table 1 Details of the experimental procedure

Days	*Solution (distilled water)*	
	A (100 ml)	*B (100 ml)*
1-10	Water	Water
11-12	Water	100 mM LiCl
13-14	Water	Water
15-16	10 mM LiCl	10 mM NaCl
17-18	Water	Water
19-20	10 mM LiCl	10 mM LiCl
21-22	10 mM NaCl	Water
23-30	Water	Water

The rats were observed alone for 10 min daily at normal room illumination in a circular arena (diam. 1 m, walls 40 cm) which was painted black and the floor divided into 21 segments by white painted lines. Behaviour recorded included the number of segments crossed, the number of times each animal reared up on the hind legs, and the time spent immobile/grooming. On days 13-14 the animals were not tested since they were disturbed for cage cleaning. A random test order was adopted.

Results

Individual fluid consumption, body weights and behavioural measures were compared daily with the previous day's figures using a Wilcoxon Matched-pairs signed-ranks test. The results are shown in Table 2 and Figure 1. Treatment days refer to the time at which intake was measured, so that behaviour observed on day 11, for example, corresponds with the consequences of lithium consumption between days 10 and 11.

There was a high correlation between mean body weight and baseline water intake ($r = 0.79$, $P < 0.001$), both increased as the study progressed.

On the first treatment (days 11-12, 100 mM LiCl) the rats consumed between 1 and 2 mM LiCl, while there was no change in water intake from previous levels. Although the corresponding behavioural measures showed a significant increase in time spent immobile/grooming there was no difference in locomotor activity or rearing frequency.

When the rats were provided with a choice between two 10 mM salt solutions for the second treatment (days 15-16) they drank between 0.1 and 0.5 mM LiCl and 0.5 to 4.8 mM NaCl. The volume of NaCl consumed was similar to the baseline water measures; although it increased on the second day, this was not significant. Behavioural measurements again demonstrated a significant increase in time spent immobile/grooming; also a significant decrease in the distance travelled and rearing frequency.

When 10 mM LiCl was offered in both bottles for the third treatment (days 19-20) the mean volume consumed increased, while the intake (0.3-0.9 mM) was actually less than for the first trial (days 11-12) when the rats could drink either a more concentrated solution of LiCl (100 mM) or water. Similar behavioural changes occurred to those seen in earlier trials, but only the decreased ambulation was significant (day 19) and there was little change between (days 19-20).

Following this LiCl presentation the rats drank

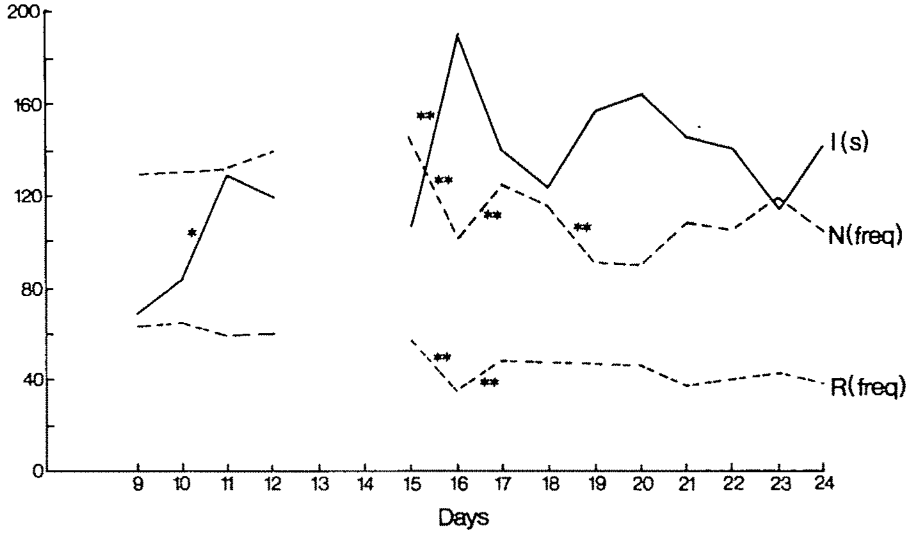

Figure 1 Median values of behaviour indices obtained between days 9-24 I = Time spent immobile; N = number of segments crossed, R = rearing frequency
* $P < 0.02$, ** $P < 0.01$ (Differs from previous measure, Wilcoxon signed-ranks matched-pairs test, two-tailed).

Table 2 Details of fluid consumption and body weight measurements

Day	*Body weight (g)*	*Water (ml)*	*10 mM NaCl (ml)*	*10 mM LiCl (ml)*	*100 mM LiCl (ml)*
1	133.0 ± 9.4	34.3 ± 1.0			
2	137.2 ± 9.3	31.2 ± 1.3			
3	140.5 ± 8.9	33.6 ± 1.2			
4	144.8 ± 9.1	34.5 ± 1.1			
5	149.1 ± 8.4	29.3 ± 1.5			
6	153.9 ± 8.8	33.1 ± 0.9			
7	160.9 ± 8 3	32 8 ± 1.1			
8	162.5 ± 7 6	33.9 ± 1.3			
9	163.4 ± 8.0	33.9 ± 1.3			
10	167.8 ± 7.6	32.0 ± 0.9			
11	173.1 ± 7.6	33.3 ± 1.1			1.5 ± 0 2
12	175 5 ± 7.4	34.5 ± 1.1			1.5 ± 0.1
13	187.4 ± 7.9	32.4 ± 0.9			
14	188.7 ± 7.5	35.7 ± 1 1			
15	187.1 ± 7.3		33.1 ± 4.9	2.2 ± 0.4	
16	188.5 ± 7.6		38.6 ± 3.6	1.3 ± 0.3	
17	192.6 ± 7.1	36.1 ± 1.2			
18	196.8 ± 7.1	34.6 ± 1.7			
19	186.6 ± 6.7			6.4 ± 0.6	
20	177.4 ± 6 6			6.1 ± 0.4	
21	199.0 ± 6.8	31.2 ± 5.7	28.0 ± 6.7		
22	202.3 ± 7.0	36.1 ± 2.4	6.0 ± 2.4*		
23	207.0 ± 6.9	38.1 ± 1 0			
24	209.9 ± 7.0	44.2 ± 1.0			
25	219.6 ± 6.5	42.5 ± 1.5			
26	221.3 ± 6.9	39.3 ± 1.3			
27	225.1 ± 6.8	37.8 ± 1.1			
28	227.8 ± 6.4	41.8 ± 1.2			
29	229.2 ± 5.9	37.4 ± 1.1			
30	234.2 ± 5.9	41.1 ± 1.7			

Values expressed as mean with s.e. mean
* Differs from previous day's measure ($P < 0.02$)

a large quantity of fluid ($\bar{X}_{total}$ = 59.2 ml) which comprised slightly more water than NaCl. However, NaCl consumption dropped sharply on the second day while water intake returned to baseline levels. There was no significant change in the behavioural measure following the final LiCl presentation.

Discussion

The return to baseline levels for both water consumption and behaviour indicates that the effects were, in fact, discrete. While this is not unexpected for the physiological response it is interesting that any stable baseline behaviour was maintained. In this sort of apparatus, once the setting ceases to be 'novel' or aversive to the rat, a decrease in overall activity is generally observed with repeated testing (Archer, 1973). Thus any reaction to extraneous stimuli (e.g., drug administration) might well be lessened as the experiment progressed (Kršiak & Janků, 1971). In the present study a significant change was noted for each of the three trials on at least one index while no significant alterations in behaviour were recorded on the eight baseline water days.

Two explanations are possible for the greater lithium consumption when water was available. Lithium causes polydipsia which may act to increase renal activity and thus lithium excretion (Smith *et al.*, 1970). Alternatively, when water was not present the rats learned to avoid both bottles, whereas when water was available in one bottle this increased the probability of sampling the wrong bottle (i.e., lithium) since these were switched over each day.

Previous studies have used either water (Smith *et al.*, 1970) or saline (Thomsen *et al.*, 1974) for the investigation of lithium toxicity. The present work supports the view that both liquids act, presumably, to reduce the retention of lithium ions. Following the lithium-only presentation on days 19-20 the polydipsic response was composed almost equally of water and saline on day 21 while on day 22 saline consumption was significantly decreased. In view of previous findings that rats will consume enough saline to compensate for renal sodium losses (Thomsen *et al.*, 1974) the optimal saline concentration was voluntarily achieved. Further investigation of saline/water choices when lithium is chronically administered in food or by injection is planned.

Whilst saline and water both act to reduce lithium toxicity, both were ineffective in preventing the behavioural effects of the salt. In fact the greatest depressant effect was observed on the lithium/sodium trials (days 15-16) when the least lithium was consumed.

Provision for voluntary consumption of saline may be useful for behavioural studies since chronic administration of LiCl has been reported to reduce individual foot-shock 'jump response' thresholds in rats (Harrison-Read & Steinberg, 1971) and to increase aggression in the cage environment (Schreiber & Roháčová, 1971). This irritability may well account for different behaviours observed in laboratory rodents treated with lithium salts by different routes (injection, food, stomach load, drinking water) and over different periods, by removing a potential confounding variable. For example, we have noted that rats administered 3 mEq/kg LiCl (i.p.) and placed together in the home cage will often fight for several minutes (Syme & Syme, 1973). Observed physical discomfort (abdominal contractions) following injection, particularly in a social setting, may also be manifest as a 'depressant' effect of the salt.

The ability of rats to choose their own lithium intake might be applicable to studies using drug-induced 'manic' activity (Cox, Harrison-Read, Steinberg & Tomkiewicz, 1971). If rats can show an 'antidotal thirst' for water or saline to counteract lithium toxicity, they may also be able to monitor their consumption of lithium solutions to reduce an adverse drug response. Perhaps this would be best demonstrated in groups of caged mice, where such hyperactivity is associated (Chance, 1946) with high mortality.

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(Received May 13, 1975.
Revised July 18, 1975.)

Br. J. Pharmac. (1975), 55, 415-422

EFFECTS OF MONOAMINE OXIDASE INHIBITION BY CLORGYLINE, DEPRENIL OR TRANYLCYPROMINE ON 5-HYDROXYTRYPTAMINE CONCENTRATIONS IN RAT BRAIN AND HYPERACTIVITY FOLLOWING SUBSEQUENT TRYPTOPHAN ADMINISTRATION

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1 The effect of various doses of tranylcypromine on the degree of inhibition of rat brain monoamine oxidase (MAO) using 5-hydroxytryptamine (5-HT), dopamine and phenylethylamine as substrates has been examined 120 min after injection of the inhibitor. The concentration of brain 5-HT was also examined both after tranylcypromine alone and also when L-tryptophan (100 mg/kg) had been given 30 min after the tranylcypromine.

2 All doses of tranylcypromine greater than 2.5 mg/kg totally inhibited MAO oxidation of 5-HT, phenylethylamine and dopamine as measured *in vitro* and produced a similar rise of brain 5-HT *in vivo* When tryptophan was also given, there was a further rise of brain 5-HT, which was comparable after all doses of tranylcypromine above 2.5 mg/kg and the characteristic syndrome of hyperactivity made its appearance.

3 Clorgyline (a 'Type A' MAO inhibitor), in doses up to 10 mg/kg, did not totally inhibit MAO activity towards phenylethylamine although it did inhibit 5-HT oxidation by 100%. Deprenil (a 'Type B' MAO inhibitor) at doses up to 10 mg/kg did not fully inhibit 5-HT oxidation although phenylethylamine oxidation was inhibited almost completely. Administration of either compound alone did not produce as great an accumulation of brain 5-HT as that seen after tranylcypromine (2.5 mg/kg) and subsequent administration of tryptophan did not cause hyperactivity or the rise of brain 5-HT seen after tranylcypromine (2.5 mg/kg) plus tryptophan.

4 Administration of clorgyline plus deprenil (2.5 mg/kg of each) almost totally inhibited oxidation of both 5-HT and phenylethylamine; subsequent tryptophan administration resulted in a rise of brain 5-HT nearly as great as that seen following tranylcypromine (2.5 mg/kg) plus tryptophan and the animals became hyperactive.

5 No evidence was found pointing to the formation of any other 5-substituted indole in the brain following tranylcypromine plus L-tryptophan administration as suggested by others.

6 It is concluded that while 5-HT may normally be metabolized in the brain by 'Type A' MAO *in vivo,* when this form is inhibited, 5-HT can still be metabolized by 'Type B' enzyme. It is only when both forms are almost totally inhibited that the largest rise of brain 5-HT is seen and subsequent tryptophan administration produces the hyperactivity syndrome.

Introduction

Administration of tryptophan to rats pretreated with the monoamine oxidase (MAO) inhibitor, tranylcypromine, results in a characteristic hyperactivity syndrome (Grahame-Smith, 1971). The evidence suggests that the hyperactivity is the result of increased 5-hydroxytryptamine (5-HT) synthesis with spill over on to post-synaptic receptors, and inhibition of MAO appears to be an essential factor for the production of the syndrome. Different forms of MAO have been demonstrated both *in vitro* and *in vivo* (see Sandler & Youdim, 1972 for review). Using clorgyline, Johnston (1968) distinguished between two enzyme systems, 'Type A' and 'Type B'. Type A is sensitive to inhibition by clorgyline and oxidatively deaminates 5-hydroxytryptamine, noradrenaline and dopamine. Type B is more resistant to clorgyline but is inhibited by deprenil and oxidizes phenylethylamine and dopamine but not 5-HT (Sandler & Youdim, 1974; Houslay & Tipton, 1974). It thus seemed reasonable to predict that if the hyperactivity syndrome were due to 5-HT alone, then hyperactivity would appear after tryptophan administration to rats pretreated with clorgyline but not deprenil. However, Squires & Buus Lassen (1975) reported that tryptophan after clorgyline did not produce hyperactivity whereas it was seen if tryptophan followed clorgyline plus deprenil.

We have now investigated the reasons for the absence of hyperactivity following clorgyline alone by studying brain 5-HT and tryptophan concentrations and brain MAO activity following tranylcypromine, clorgyline and deprenil administration and correlated these changes with the appearance of the behavioural changes.

A preliminary report has been given of some of these findings (Green & Youdim, 1975).

Methods

Male Sprague-Dawley rats (160-200 g) (Anglia Laboratory Animals, Alconbury, Huntingdon) were used in all experiments. Tranylcypromine (Smith, Kline & French, Ltd.), clorgyline (May & Baker, Ltd.) or deprenil were dissolved in 0.9% w/v NaCl solution (saline) and injected intraperitoneally in various doses to groups of 6 rats. After 30 min, L-tryptophan (100 mg/kg i.p.) was given to 3 rats and saline to the other three. After a further 90 min, the rats were killed, the brains removed and divided along the mid-line. One half was homogenized in acidified butanol (Chang, 1964); 5-HT was measured by the method of Curzon & Green (1970) and tryptophan by that of Denckla & Dewey (1967). The other half was homogenized in 0.32 M sucrose and MAO activity towards the substrates [1-^{14}C]-dopamine, [1-^{14}C]-5-hydroxytryptamine (both from the Radiochemical Centre, Amersham) and [1-^{14}C]-phenylethylamine (NEN Chemicals, GmbH) was measured by the method of Southgate & Collins (1969). Protein was estimated by the procedure of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as standard. All enzyme activities were calculated as nmol deaminated product formed/mg protein per 30 min incubation and results are expressed as the mean ± s.e.mean of the percentage inhibition compared to saline-injected controls. For analysis of variance of these groups, arcsin transformation of the percentage inhibition values was performed (Snedecor & Cochran, 1967) before analysis.

Activity was measured on both groups of 3 animals for 120 min after MAO inhibition using Animex activity meters (sensitivity and tuning settings: 30 μA) as described previously (Grahame-Smith, 1971; Green & Grahame-Smith, 1974).

Results

Effect of L-tryptophan on rat brain 5-hydroxytryptamine, tryptophan and monoamine oxidase activity

Initial experiments were performed to see whether L-tryptophan administration altered MAO activity. Rats were injected with saline and 30 min later given either saline or L-tryptophan (100 mg/kg). After a further 90 min they were killed and brain 5-HT, tryptophan and MAO activity measured. After L-tryptophan, there was a large increase in brain tryptophan concentrations and an increase in 5-HT (Table 1). Tryptophan did not inhibit MAO activity towards any of the substrates examined and no increased behavioural activity was observed.

Effect of various doses of tranylcypromine followed by L-tryptophan on hyperactivity and brain 5-hydroxytryptamine, tryptophan and MAO activity

Rats were injected with 1.0, 2.5, 5.0 and 10 mg/kg tranylcypromine and the experiment continued as described in the methods section. None of the dose of tranylcypromine alone produced hyper-

Table 1 Effect of L-tryptophan (100 mg/kg) on brain tryptophan and 5-hydroxytryptamine (5-HT) concentrations 90 min later

Injected	*Brain tryptophan (μg tryptophan/g (wet wt))*	*Brain 5-hydroxytryptamine (μg 5-HT/g brain (wet wt))*
Saline	4.71 ± 0.38 (3)	0.49 ± 0.01 (10)
L-tryptophan (100 mg/kg)	35.0 ± 0.59 (3)	0.81 ± 0.05 (3)

Results of brain 5-HT and tryptophan concentrations expressed as mean ± s.e. mean with number of determinations in brackets.

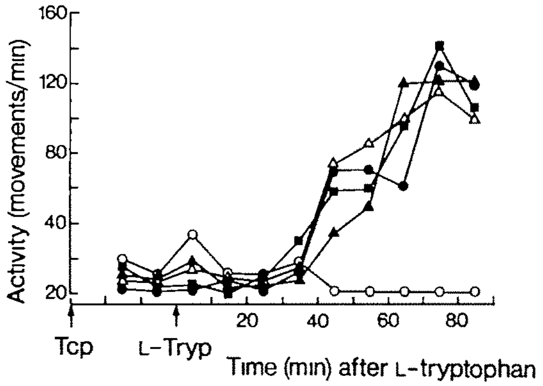

Figure 1 Effect of L-tryptophan (100 mg/kg) injection following various doses of tranylcypromine on hyperactivity. Rats were injected with tranylcypromine and with L-tryptophan (100 mg/kg) 30 min later. Activity measured as movements/minute. Tranylcypromine doses: 1.0 mg/kg (○), 2.5 mg/kg (●), 5.0 mg/kg (△), 10.0 mg/kg (▲), 20.0 mg/kg (■).

activity nor was there any change when tryptophan (100 mg/kg) was given after the 1 mg/kg dose of tranylcypromine. However, tryptophan plus tranylcypromine at a dose of 2.5 mg/kg or more resulted in the appearance of the hyperactivity syndrome (Grahame-Smith, 1971). The degree of hyperactivity was then independent of the dose of tranylcypromine (Figure 1).

Biochemical measurements showed that there was only a small increase in brain 5-HT concentration following tranylcypromine (1 mg/kg). When L-tryptophan was given after this dose of tranylcypromine, brain 5-HT concentration was no higher 90 min later than that seen when L-tryptophan alone had been given (Table 2). With this dose of tranylcypromine MAO activity was inhibited by 86% and 85% with 5-HT and phenylethylamine respectively as substrates. At the higher doses of tranylcypromine, both forms of the enzyme are totally inhibited (Table 2).

The dose of 20 mg/kg tranylcypromine used in previous investigations of hyperactivity (for review see Green & Grahame-Smith, 1975) only produces a similar degree of hyperactivity to that seen after lower doses (Figure 1). A study of the time course of alterations in brain 5-HT and tryptophan concentrations was made. Brain 5-HT increases linearly with time following tranylcypromine (20 mg/kg) with a much steeper rate of increase after L-tryptophan (100 mg/kg). Brain tryptophan concentrations following L-tryptophan (100 mg/kg) increased rapidly to reach a maximum 60 min later, followed by a fairly rapid decline in the next 30 min (Figure 2).

Effect of various doses of clorgyline or deprenil followed by L-tryptophan on hyperactivity and brain 5-hydroxytryptamine and MAO activity

Rats were injected with 1.0, 2.5, 5.0 and 10.0 mg/kg clorgyline or deprenil and the experiment continued as described in the methods section. None of the doses of clorgyline resulted in the hyperactivity syndrome, even after further administration of tryptophan (Figure 3). Essentially complete inhibition of 5-HT oxidation by MAO was produced by 2.5 mg/kg or more of clorgyline whereas phenylethylamine oxidation was not strongly inhibited even by 10 mg/kg clorgyline (Table 3).

After deprenil, hyperactivity was not seen at doses up to 5.0 mg/kg, even when tryptophan was given (Figure 4). However, following the 10 mg/kg

Table 2 Effect of various doses of tranylcypromine with or without L-tryptophan injection (100 mg/kg) 30 min later on brain 5-hydroxytryptamine (5-HT) concentrations and monoamine oxidase (MAO) activity towards 5-HT, dopamine and phenylethylamine 120 min after initial injection

Dose of tranylcypromine (mg/kg)	*Brain 5-hydroxytryptamine (μg 5-HT/g brain (wet wt))*		*% Inhibition of MAO activity towards substrates*		
	Saline	*L-tryptophan (100 mg/kg)*	*5-HT*	*Dopamine*	*Phenylethylamine*
1.0	0.60 ± 0.03 (3)	0.75 ± 0.14 (3)	86 ± 5 (6)	86 ± 1 (6)	85 ± 1 (3)
2.5	0.78 ± 0.02 (3)	1.12 ± 0.14 (3)	100 (6)	100 (6)	100 (6)
5.0	0.77 ± 0.02 (3)	1.16 ± 0.06 (3)	100 (6)	100 (6)	100 (6)
10.0	0.79 ± 0.05 (3)	1.18 ± 0.07 (3)	100 (6)	100 (6)	N.D.

Results of brain 5-HT shown 120 min after injection of inhibitor when saline or L-tryptophan (100 mg/kg) had also been given 30 min after tranylcypromine. % Inhibition of MAO activity also shown 120 min after injection of tranylcypromine. Results expressed as mean ± s.e. mean with number of determinations in brackets N.D.: Not determined.

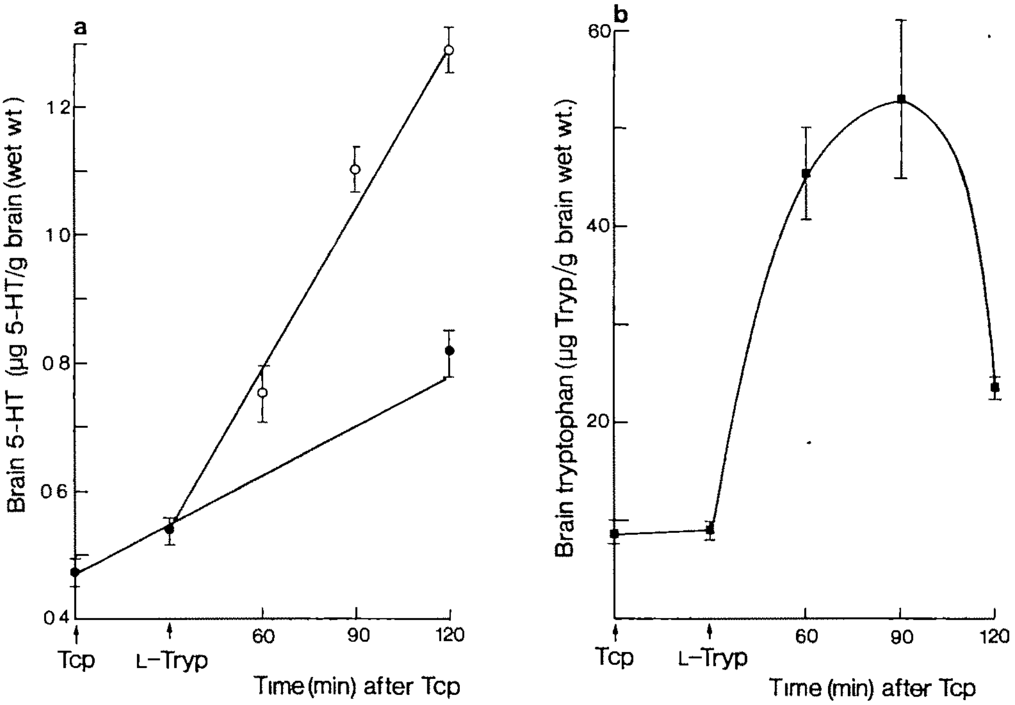

Figure 2 Effect of L-tryptophan (L-Tryp) (100 mg/kg) following tranylcypromine (Tcp) (20.0 mg/kg) on rat brain 5-hydroxytryptamine (5-HT) and tryptophan concentrations (a) Effect on brain 5-HT concentrations. Brain 5-HT concentrations following tranylcypromine 20 mg/kg (●) and following L-tryptophan 30 min after tranylcypromine (○). (b) Effect of tranylcypromine 20 mg/kg with L-tryptophan 100 mg/kg 30 min later on brain tryptophan (■).

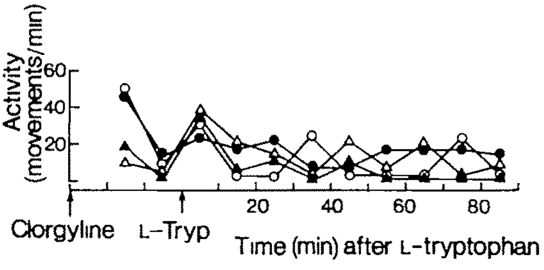

Figure 3 Effect of L-tryptophan (L-Tryp) (100 mg/kg) injection following various doses of clorgyline on hyperactivity. Rats were injected with L-tryptophan (100 mg/kg) 30 min later. Activity measured as movements/minute. Clorgyline doses 1.0 mg/kg (○), 2.5 mg/kg (●), 5.0 mg/kg (△), 10.0 mg/kg (▲).

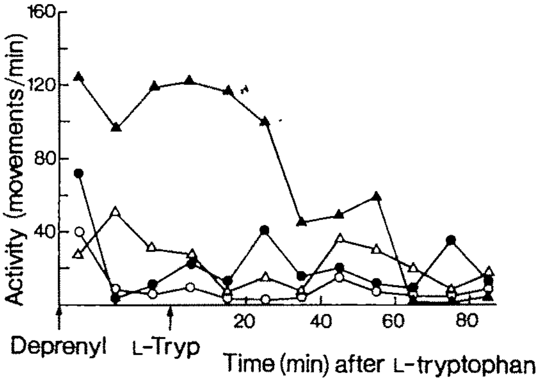

Figure 4 Effect of L-tryptophan (100 mg/kg) injection following various doses of deprenil on hyperactivity. Rats were injected with deprenil and with L-tryptophan (100 mg/kg) 30 min later. Activity was measured as movements/minute. Deprenil doses 1.0 mg/kg (○), 2.5 mg/kg (●), 5.0 mg/kg (△), 10.0 mg/kg (▲).

dose, much spontaneous locomotor activity was measured on the meters, which was not accompanied by any of the other behavioural changes associated with the hyperactivity syndrome (for details of these changes, see Grahame-Smith, 1971) and not altered by L-tryptophan administration. None of the doses of deprenil when given with tryptophan resulted in a rise in brain 5-HT concentration greater than that seen in animals not given deprenil. Nor did deprenil at any dosage schedule inhibit MAO activity towards 5-HT by more than 50% although phenylethylamine oxidation was almost totally inhibited by 5.0 mg/kg deprenil (Table 3).

Effect of various doses of deprenil plus clorgyline followed by L-tryptophan on hyperactivity, brain tryptophan, 5-hydroxytryptamine and MAO activity

An attempt was made to confirm the finding of Squires & Buus Lassen (1975) that when deprenil and clorgyline are given in combination, hyperactivity follows the subsequent administration of tryptophan. Experimental procedures were as before except that the two inhibitors were given together. While no hyperactivity was observed when 1 mg/kg of each inhibitor was given 30 min before L-tryptophan (100 mg/kg), the hyperactivity syndrome appeared when the dose of each inhibitor was increased to 2.5 mg/kg (Figure 5). 5-HT and phenylethylamine oxidation were only inhibited by more than 90% at the higher dose, whilst 5-HT concentrations only rose to about those observed after tranylcypromine (2.5 mg/kg) at the same dosage combination (Table 4).

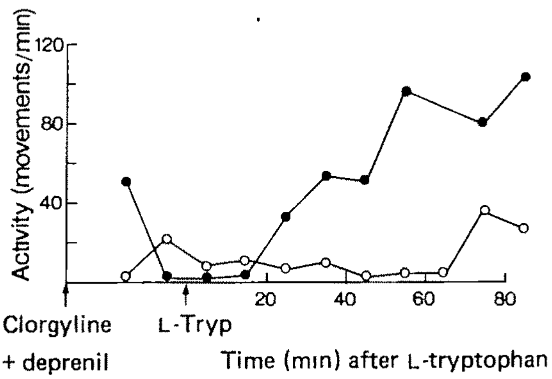

Figure 5 Effect of L-tryptophan (L-Tryp) (100 mg/kg) injection following various doses of clorgyline plus deprenil on hyperactivity. Rats were injected with clorgyline plus deprenil and with L-tryptophan (100 mg/kg) 30 min later. Activity measured as movements/minute. Clorgyline (1.0 mg/kg) + deprenil (1.0 mg/kg) (○), clorgyline (2.5 mg/kg) + deprenil (2.5 mg/kg) (●).

Measurement of brain 5-HT by two methods after tranylcypromine and L-tryptophan

Squires (1975) suggested that hyperactivity is seen only when clorgyline and deprenil are given together because it stems from the action of a

Table 3 Effect of various doses of clorgyline or deprenil with or without L-tryptophan injection (100 mg/kg) 30 min later on brain 5-hydroxytryptamine (5-HT) concentrations and monoamine oxidase (MAO) activity towards 5-HT, dopamine and phenylethylamine 120 min after initial injection

Injected	*Dose of deprenil or clorgyline (mg/kg)*	*Brain 5-hydroxytryptamine (µg 5-HT/g brain (wet wt))* *Saline*	*L-tryptophan (100 mg/kg)*	*% Inhibition of MAO activity towards substrates* *5-HT*	*Dopamine*	*Phenylethylamine*
Clorgyline	1.0	0.60 ± 0.06 (9)	0.90 ± 0.10 (6)	61 ± 7 (11)	51 ± 6 (12)	19 ± 7 (3)
	2.5	0.67 ± 0.06 (9)	0.96 ± 0.11 (6)	88 ± 6 (12)	71 ± 10 (12)	24 ± 9 (3)
	5.0	0.65 ± 0.04 (9)	0.91 ± 0.10 (6)	98 ± 1 (12)	87 ± 5 (12)	29 ± 10 (3)
	10.0	0.69 ± 0.03 (6)	0.90 ± 0.09 (6)	99 ± 1 (9)	96 ± 18 (9)	67 ± 8 (3)
Deprenil	1.0	0.51 ± 0.01 (9)	0.73 ± 0.09 (6)	23 ± 7 (12)	28 ± 5 (9)	58 ± 5 (3)
	2.5	0.58 ± 0.03 (9)	0.72 ± 0.02 (6)	29 ± 6 (12)	32 ± 6 (12)	79 ± 1 (3)
	5.0	0.56 ± 0.03 (9)	0.77 ± 0.07 (6)	31 ± 4 (12)	43 ± 4 (12)	91 ± 4 (3)
	10.0	0.57 ± 0.02 (6)	0.78 ± 0.12 (6)	44 ± 8 (9)	57 ± 5 (9)	N.D.

Results of brain 5-HT shown 120 min after injection of inhibitor when saline or L-tryptophan (100 mg/kg) had also been given 30 min after inhibitor. % Inhibition of MAO activity also shown 120 min after injection of inhibitor. Results expressed as mean ± s.e. mean with number of determinations in brackets N.D.: Not determined.

5-HT metabolite metabolized by Type B MAO which is being measured as 5-HT in the brain. Certainly the method of measuring 5-HT concentration by an *o*-phthalaldehyde (OPT) reaction used in this study (Curzon & Green, 1970) and that of Squires & Buus Lassen (1975) is somewhat non-specific, for it will measure other 5-substituted indoles (Maickel & Miller, 1966). Since 5-methoxytryptamine is present in the hypothalamus (Green, Koslow & Costa, 1973), produces a strong fluorescent reading with OPT and gives rise to behavioural changes similar but not identical to those seen following increased 5-HT synthesis (Green, Hughes & Tordoff, 1975), it was important to ascertain that the apparent rise of 5-HT seen after tranylcypromine and tryptophan does not derive spuriously from this or another 5-substituted pharmacologically active indoleamine.

Rats were injected with tranylcypromine (20 mg/kg) followed by L-tryptophan (100 mg/kg) 30 min later. After a further 60 min, the rats were killed, the brains divided down the mid-line and 5-HT measured in one half by the OPT method (Curzon & Green, 1970) and in the other by the ninhydrin method of Snyder, Axelrod & Zweig (1965) which at the appropriate wave length appears to measure only 5-HT (Snyder *et al.*, 1965). As they were so similar, the results suggested that the OPT method was measuring only 5-HT (Table 5) and that no other 5-substituted indoles were present.

Discussion

In agreement with Squires & Buus Lassen (1975), we found that tryptophan administration to rats pretreated with either clorgyline or deprenil alone does not result in the hyperactivity syndrome, which was, however, observed after the administration of both inhibitors at low doses. Squires (1975) has suggested that this hyperactivity derives from the formation of an *N*-substituted derivative of 5-HT which is deaminated by Type B MAO to 5-hydroxyindoleacetic acid. However, our results lead us to quite different conclusions.

Pretreatment of rats with tranylcypromine at a dose of 2.5 mg/kg or more, totally inhibited phenylethylamine and 5-HT metabolism by MAO as measured *in vitro*. All doses of this magnitude or greater caused a similar 5-HT rise, measured as a

Table 4 Effect of various doses of clorgyline plus deprenil with or without L-tryptophan injection (100 mg/kg) 30 min later on brain 5-hydroxytryptamine (5-HT) concentrations and monoamine oxidase (MAO) activity towards 5-HT, dopamine and phenylethylamine 120 min after initial injection

Dose of clorgyline plus deprenil (mg/kg) of each)	*Brain 5-hydroxytryptamine (μg 5-HT/g brain (wet wt))*		*% Inhibition of MAO activity towards substrates*		
	Saline	*L-tryptophan (100 mg/kg)*	*5-HT*	*Dopamine*	*Phenylethylamine*
1.0 + 1.0	0.68 ± 0.03 (9)	0.80 ± 0.08 (3)	84 ± 7 (10)	83 ± 4 (12)	76 ± 20 (3)
2.5 + 2.5	0.76 ± 0.05 (8)	1.04 ± 0.14 (3)	97 ± 14 (12)	99 ± 1 (12)	87 ± 13 (3)

Results of brain 5-HT shown 120 min after injection of inhibitor when saline or L-tryptophan (100 mg/kg) had also been given 30 min after the inhibitor. % Inhibition of MAO activity also shown 120 min after injection of the inhibitor. Results expressed as mean ± s.e. mean with number of determinations in brackets

Table 5 Concentration of brain 5-hydroxytryptamine (5-HT) measured by two methods following tranylcypromine and L-tryptophan administration

	Brain 5-hydroxytryptamine (μg 5-HT/g brain (wet wt))	
	Ninhydrin method	*OPT method*
Saline	0.49 ± 0.01 (4)	0.49 ± 0.02 (4)
Tranylcypromine (20 mg/kg) + L-tryptophan (100 mg/kg)*	1.32 ± 0.06 (6)	1.24 ± 0.10 (6)

* Tranylcypromine given 30 min before L-tryptophan with measurement of brain 5-HT 60 min later.

rate of 5-HT synthesis or 5-HT accumulation when tryptophan was also administered. In contrast, clorgyline at doses above 2.5 mg/kg, while totally inhibiting MAO activity to 5-HT, did not produce a greater than 70% inhibition of phenylethylamine oxidation (Table 3). Pretreatment with deprenil did not inhibit 5-HT oxidation by more than 50% even at a dose of 10 mg/kg. None of the dosage levels of either clorgyline or deprenil resulted in a rise of 5-HT concentration comparable to that observed after tranylcypromine (2.5 mg/kg) nor was 5-HT accumulation after subsequent tryptophan administration as great as that seen after tranylcypromine (2.5 mg/kg) plus tryptophan.

In view of the limited and specific distribution pattern of the clorgyline-insensitive 5-HT oxidizing form of MAO recently reported (Gascoigne, Williams & Williams, 1975), it seems unlikely that this enzyme is responsible for the continuing metabolism of 5-HT following clorgyline injection which is seen in our experiments.

Deprenil and clorgyline (1 mg/kg of each), when injected together, also failed to inhibit totally both forms of the enzyme. However 2.5 mg/kg of each almost totally inhibited both forms of the enzyme. Brain 5-HT also rose to the concentration seen after administration of tranylcypromine (2.5 mg/kg), with a further similar increase after tryptophan.

These results suggest that while 5-HT may normally be predominantly metabolized *in vivo* by Type A monoamine oxidase, when it is inhibited (e.g. by clorgyline), Type B MAO continues to act on the amine. Thus 5-HT concentrations rise more slowly after clorgyline than after the non-specific inhibitor tranylcypromine. When deprenil is given, of course, Type A MAO is only partially inhibited. These findings strengthen the view (Youdim, 1973) that MAO acts *in vivo* as an integrated enzyme system with properties which differ from the individual enzyme forms studied *in vitro.*

The results relating to hyperactivity demonstrate that it is only when both forms of MAO are almost totally inhibited, as when clorgyline and deprenil are given together, that the phenomenon is seen. Only then, presumably, is 5-HT not being oxidized in the presynaptic compartment by intraneuronal MAO and is thus able to 'spill-over' into functional activity. Tranylcypromine (1 mg/kg) inhibited 5-HT oxidation by 86% and phenylethylamine oxidation by 85% but even so it did not cause either hyperactivity or the rise of 5-HT concentration seen after the larger doses which totally inhibited the enzyme. This finding suggests that amounts of MAO are present in the brain grossly in excess of normal requirements. If true, this may be one reason that successful therapy of depressive states by monoamine oxidase inhibitors is difficult to achieve Previous studies on human brain have indicated that MAO activity towards various substrates is not normally inhibited much above 70% by therapeutic doses of inhibitors (Youdim, Collins, Sandler, Bevan-Jones, Pare & Nicholson, 1972).

Our results pertaining to the inhibition of dopamine oxidation after either clorgyline or deprenil indicate that dopamine is metabolized both by Type A and Type B MAO in agreement with the report by Yang & Neff (1974).

Analysis of 5-HT in rats treated with tranylcypromine (20 mg/kg) and L-tryptophan using the *o*-phthalaldehyde and ninhydrin methods gave essentially similar results (Table 5). Squires (1975) suggested that an N-methylated derivative might be formed following MAO inhibition and L-tryptophan administration, although it would be indistinguishable from 5-HT using the OPT method. Thus results with the OPT method would have been higher than those with ninhydrin, as the former measures any 5-substituted indole (Maickel & Miller, 1966). However, the similarity between the two sets of results reported here argues against involvement of any other 5-substituted indole, as does the observation that the brain 5-HT rise is smaller after clorgyline (even at a dose of 10 mg/kg) than after tranylcypromine (2.5 mg/kg), pointing to incomplete inhibition of 5-HT degradation. Nor does the rise of 5-HT concentrations seen after injection of either clorgyline plus deprenil (2.5 mg/kg of each) or tranylcypromine (2.5 mg/kg) appear to be due to formation of a 5-HT derivative, as the value obtained when translated into a rate of 5-HT synthesis agrees well with those calculated by other methods not requiring the use of a MAO inhibitor (Neff & Tozer, 1968).

The rapid decrease in brain typtophan observed 90 min after tranylcypromine injection is probably due to the induction of hepatic tryptophan pyrrolase activity by its substrate, leading to rapid peripheral metabolism of tryptophan. This in turn would presumably decrease brain tryptophan. Recent evidence suggests that variations in activity of this enzyme can alter brain tryptophan concentrations (Green, Sourkes & Young, 1975; Green, Woods, Knott & Curzon, 1975).

Thus while the involvement of any other indole cannot totally be excluded, we nevertheless feel that the most plausible explanation for the results obtained by Squires & Buus Laasen (1975) and ourselves is also the simplest, that unless both Type A and Type B MAO are almost totally inhibited, hyperactivity will not occur; while 5-HT may normally be metabolized by Type A enzyme *in vivo,* when this form is blocked, oxidation can be continued by Type B enzyme. If this hypothesis is correct, it suggests that increasing the functional activity of 5-HT by using selective inhibitors may be difficult to achieve.

We gratefully acknowledge the excellent technical assistance of Mr B.D. Mitchell, Miss Ann F.C. Tordoff and Miss Janet P. Hughes. We also thank Smith, Kline & French Ltd. and May & Baker Ltd. for a supply of tranylcypromine and clorgyline respectively, and Professor J. Knoll (Budapest, Hungary) for a gift of deprenil. Finally, we thank Professor D.G. Grahame-Smith for his helpful comments.

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(Received May 29, 1975.
Revised June 26, 1975.)

Br. J. Pharmac. (1975), 55, 423-430

RESPONSE OF RAT LUNG TO HUMORAL MEDIATORS OF ANAPHYLAXIS AND ITS MODIFICATION BY DRUGS AND SENSITIZATION

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1 The effects of humoral mediators of anaphylaxis on the bronchial function of the pithed rat have been assessed and compared with their effects in the guinea-pig and man.

2 5-Hydroxtryptamine, acetylcholine and bradykinin were bronchoconstrictor. Slow reacting substance in anaphylaxis and prostaglandin $F_{2\alpha}$ caused only slight constriction at high doses and histamine was not active.

3 Bradykinin was less active in the rat than in the guinea-pig and its effects were not antagonized by anti-inflammatory analgesic drugs.

4 β-Adrenoceptor blockade potentiated the activity of bradykinin but not that of 5-hydroxytryptamine or acetylcholine.

5 Dexamethasone reduced the activity of bradykinin but not that of 5-hydroxytryptamine or acetylcholine.

6 Sensitization with *Nippostrongylus brasiliensis* did not increase the sensitivity of the rat lung to the effects of mediators.

Introduction

An animal model of bronchial asthma should parallel the human reaction in as many respects as possible. The two main animal models used, bronchial anaphylaxis in the rat and the guinea-pig, have aspects which both resemble and differ from those in man.

In the rat, allergic reactions are, as in man, initiated primarily by the combination of antigen with IgE antibody (Johansson, 1967; Ogilvie, 1967; Ishizaka & Ishizaka, 1968, Stechschulte, Orange & Austen, 1970). Systemic challenge results in an anaphylactic reaction which is characterized by hypotension and death following engorgement of the heart and small intestine, although respiratory distress has been reported (Sanyal & West, 1958; Ogilvie, 1967). Measurement of bronchial function in anaesthetized rats has revealed that anaphylactic bronchoconstriction is mediated mainly by 5-hydroxytryptamine (Church, Collier & James, 1972).

In the guinea-pig, combination of antigen with IgG antibody is primarily responsible for the initiation of the allergic response (Benacerraf, 1968). The ensuing anaphylactic reaction affects primarily the respiratory tract (Herxheimer, 1952) where histamine, slow reacting substance in anaphylaxis (SRS-A) and kinins are the main mediators (Collier & James 1966, 1967).

This paper describes the response of the rat lung to some of the mediators implicated in anaphylaxis and compares these responses with those reported for guinea-pig and human lung. Modification by specific antagonists, propranolol, dexamethasone and sensitization with *Nippostrongylus brasiliensis* of the rat bronchial responses to mediators is described.

Methods

Materials

The following substances were used: acetylcholine bromide, atropine sulphate, bradykinin (Nicolaides & De Wald, 1961), dexamethasone sodium phosphate, histamine acid phosphate, 5-hydroxytryptamine creatinine sulphate, indomethacin, meclofenamate sodium, mepyramine maleate, methysergide bimaleate, propranolol hydrochloride, prostaglandin $F_{2\alpha}$ and slow reacting substance in anaphylaxis (SRS-A) (charcoal purified material prepared from the perfusate of isolated lungs of sensitized guinea pigs as described by Berry & Collier, 1964, and tested for freedom from histamine as described by Collier & James, 1967). All doses of all compounds were calculated in terms of free base.

Sensitization of animals

All experiments were performed on male Wistar rats weighing 200-300 g. Rats were sensitized by subcutaneous injection of 5000 larvae of *Nippostrongylus brasiliensis*. They were tested for bronchial reactivity to humoral mediators 5 weeks after sensitization when anaphylactic sensitivity is maximal (Church, 1975).

Administration of substances

Dexamethasone was injected intraperitoneally 24 h before testing. Indomethacin was injected intraperitoneally 30 min before testing. Other antagonists were administered intravenously through a jugular cannula 5 min before intravenous injection of agonists.

Measurement of bronchial function

Animals were lightly anaesthetized with ether and the brain and spinal cord destroyed by pithing The trachea was cannulated and the rat ventilated with a Starling miniature respiratory pump of stroke volume 5-7 ml at 90 strokes/minute. The side arm of the tracheal cannula was connected to a non return water valve set at a pressure of 7.5 cm of water. Tracheal flow during lung inflation was measured with a pneumotachograph and recorded on a multichannel electronic recorder. Reduction of tracheal flow, indicative of bronchoconstriction, was estimated as a percentage of the maximum possible bronchoconstriction and was measured at the time of peak effect.

Results

Histamine

Histamine injected intravenously in doses of 10 μg-1 mg caused no significant bronchospasm in groups of two to five rats (Table 1). Activity was not enhanced by pretreatment with propranolol or by sensitization with *N. brasiliensis*.

5-Hydroxytryptamine

5-Hydroxytryptamine (5-HT) given intravenously in doses of 5-20 μg to a group of five rats caused a

Table 1 The response of the rat lung to mediators of anaphylaxis

Mediator	*Dose (μg)*	*No. of animals*	*Mean reduction in tracheal flow ± s.e. (%)*
Histamine	10	2	0 ± 0
	200	2	3 ± 3
	500	5	13 ± 2
	1000	5	8 ± 3
5-HT	5	5	41 ± 7
	10	6	63 ± 5
	20	6	86 ± 3
Acetylcholine	10	5	26 ± 2
	20	6	50 ± 5
	40	5	75 ± 4
Bradykinin	25	6	14 ± 2
	50	5	27 ± 4
	100	6	51 ± 6
SRS-A	1 x 10^3	6	0 ± 0
	2 x 10^3	4	1 ± 1
	5 x 10^3	5	2 ± 2
	10 x 10^3	4	3 ± 1
	20 x 10^3	4	7 ± 2
Prostaglandin $F_{2\alpha}$	50	5	8 ± 3
Saline	1 ml	6	0 ± 0

Rats were pithed and prepared for recording of tracheal flow. Responses were measured at the time of maximal effect after intravenous administration of mediator.

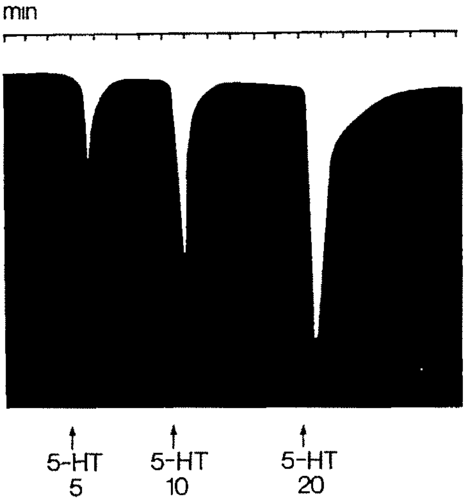

Figure 1 The effect of 5-hyrdoxytryptamine (5-HT) on the bronchial function of the rat. 5-HT, 5, 10 or 20 μg was injected intravenously into a pithed rat.

rapid but short lasting bronchoconstriction (Figure 1). This bronchospasm was linearly related to dose (Table 1). The dose of 5-HT calculated to produce a 50% constrictor response was 6.4 μg.

Methysergide, 0.1 mg/kg injected intravenously 5 min before 10 μg of 5-HT, caused a 93% reduction of response (Table 2). Atropine, 1 mg/kg, inhibited the response by 67%. Mepyramine, 2 mg/kg, and meclofenamate, 1 mg/kg, did not modify the response, The β-adrenoceptor blocking agent propranolol, 5 mg/kg, caused a small, but not significant, decrease in bronchoconstriction. Dexamethasone, 5 mg/kg injected intraperitoneally into a group of ten rats showed no effect in comparison with ten untreated animals. Sensitization 5 weeks previously with *N. brasiliensis* did not modify the response to 5-HT in a group of ten rats compared with ten unsensitized controls.

Acetylcholine

Acetylcholine injected intravenously in doses of 10-40 μg caused a marked but transient bronchospasm in a group of five rats. The response was linearly related to dose (Table 1). The dose calculated to produce a 50% constriction was 19.9 μg. Acetylcholine is, therefore, three times less spasmogenic in rat lung than is 5-HT.

Atropine, 1 mg/kg, abolished the bronchoconstrictor response to 20 μg of acetylcholine (Table 2). Methysergide, 0.1 mg/kg and mepyramine, 2 mg/kg, did not modify the bronchoconstriction. Pretreatment with propranolol, 5 mg/kg, did not potentiate the response. Dexamethasone, 5 mg/kg, decreased the response to acetylcholine by 24% in a group of ten rats but this was not significantly different from untreated controls. Sensitization

Table 2 The modification by drugs and sensitization of 5-hydroxytryptamine, acetylcholine and bradykinin-induced bronchospasm in the rat

		Bronchoconstriction (%)					
	Dose	*5-HT*		*Acetylcholine*		*Bradykinin*	
Treatment	*(mg/kg)*	*Control*	*Treated*	*Control*	*Treated*	*Control*	*Treated*
Methysergide	0.1	58	4*	43	47	74	74
Atropine	1	67	22*	57	0*	—	—
Mepyramine	2	60	57	63	64	38	35
Meclofenamate	1	54	53	—	—	30	30
Propranolol	5	59	48	59	63	46	61*
Dexamethasone	5	63	60	50	38	51	28*
Sensitization	—	63	59	50	62	51	51

The results were obtained in groups of 3-10 rats pithed and prepared for recording of tracheal flow. Methysergide, atropine, mepyramine, meclofenamate and propranolol were given intravenously 5 min before constrictor agent. Dexamethasone was given intraperitoneally 24 h before test. Sensitization was with 5,000 *N. brasiliensis* larvae injected subcutaneously five weeks before test. 5-HT, 10 μg, acetylcholine, 20 μg, and bradykinin, 100 μg, were given intravenously. The bronchoconstriction was measured at the time of maximum effect. The levels of bronchoconstriction in bradykinin treated animals have been corrected for tachyphylaxis as described in the text.

*Significantly ($P < 0.001$) different from control. — Not tested.

with *N. brasiliensis* increased bronchospasm by 24% in a group of five rats. This increase was not statistically significant.

Bradykinin

Bradykinin injected intravenously in doses of 25-100 μg caused a more slowly developing bronchoconstriction in a group of five rats (Figure 2). The bronchospasm was linearly related to dose (Table 1). The dose calculated to produce a 25% response was 41 μg.

Two characteristics of the rat bronchoconstrictor response to bradykinin were observed. Firstly, tachyphylaxis to bradykinin developed with repeated doses (Figure 2). Secondly, after a constrictor response to bradykinin the lung did not reinflate to its original volume. Reinflation of the lung at a higher pressure restored the tracheal flow to near its original value but did not alter the tachyphylaxis (Figure 2, Table 3). When assessing the effects of drugs on the bradykinin response allowance for the developing tachyphylaxis was made. In these experiments each animal received five doses of bradykinin. The mean of the responses to the second and third doses was taken as the predosed level of bronchoconstriction and the mean of the responses to the fourth and fifth doses as the bronchoconstriction following treatment. Drug or saline (0.9% w/v NaCl solution) was given between the third and fourth doses. A correction factor, calculated in control animals (Table 3), was then applied to drug-treated animals to compensate for tachyphylaxis.

Methysergide, 0.1 mg/kg, mepyramine, 2 mg/kg, and meclofenamate, 1 mg/kg, did not inhibit the bronchoconstrictor response induced by 100 μg of bradykinin (Table 2). Propranolol, 5 mg/kg, potentiated the response by 33%. This increase was statistically significant ($P < 0.001$).

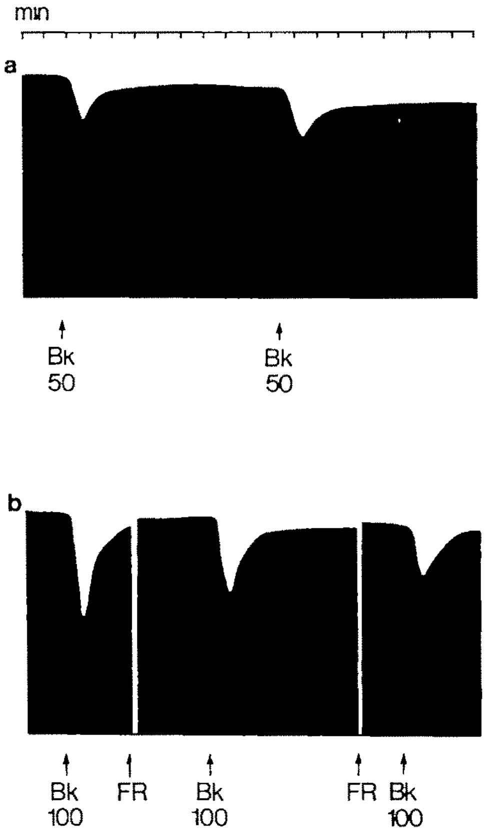

Figure 2 The effect of repeated doses of bradykinin on the bronchial function of the rat. (a) The effect of repeated injections of bradykinin (Bk) 50 μg, given intravenously to a pithed rat. (b) The effect of repeated injections of bradykinin, 100 μg, given intravenously to a pithed rat in which the lungs were forcibly reinflated (FR) between injections.

Table 3 Tachyphylaxis of rat bronchoconstriction to bradykinin

	Dose No.				
	1	*2*	*3*	*4*	*5*
Decrease in tracheal flow (%)	50	52	40	32	31
Mean		46		31	
Correction factor for tachyphylaxis			$^{46}/_{31}$ = 1 48		

Rats were pithed and prepared for recording of tracheal flow. Responses to a constant dose of 100 μg bradykinin given every 4 min, were measured at the time of maximum effect. Each response is the mean of those obtained in four animals.

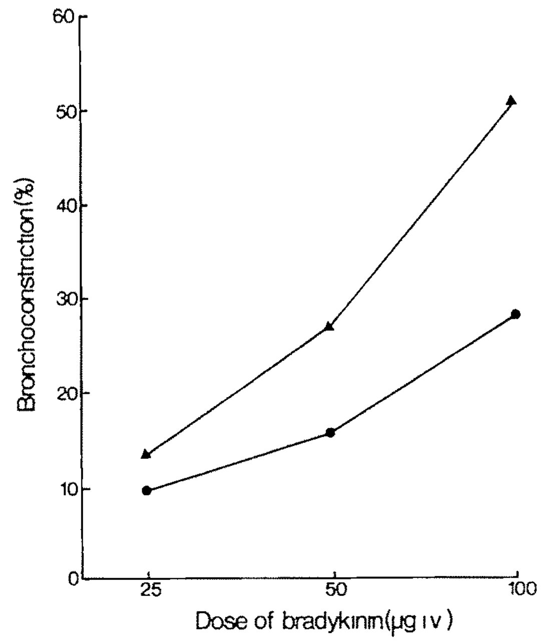

Figure 3 The effect of dexamethasone on bradykinin-induced bronchoconstriction in the rat. Dexamethasone, 5 mg/kg, (●) or saline, control (▲) was injected intraperitoneally 24 h before the rats were pithed and prepared for recording of tracheal flow. Each point is the mean of results obtained in ten rats.

Dexamethasone, 5 mg/kg, injected intraperitoneally into a group of ten rats 24 h previously inhibited the spasmogenic effects of bradykinin compared with undosed controls. Dexamethasone was more active against higher doses of bradykinin than against smaller doses (Figure 3). Sensitization with *N. brasiliensis* 5 weeks previously did not modify bradykinin-induced bronchoconstriction.

Slow reacting substance in anaphylaxis (SRS-A)

SRS-A injected intravenously in doses of 1-20 mg produced little effect on the rat lung. Only the highest dose used, 20 mg, produced a significant ($P < 0.05$) bronchospasm (Table 1). The response to SRS-A reached a maximum intensity 30 s after injection and was over by 1.5 minutes.

Propranolol, 5 mg/kg, increased the bronchospasm induced by 20 mg of SRS-A from 7% to 15%. Propranolol also extended the time course of the reaction, the maximum intensity occurring 1-2 min after dosage and being over by 4-5 minutes. Meclofenamate, 1 mg/kg, administered to three propranolol pretreated rats, abolished the SRS-A response in one animal but only slightly reduced the response in the other two rats. Because of the weak activity of SRS-A, the validity of the results obtained with meclofenamate may be questioned. No further experiments with antagonists were performed due to the relative ineffectiveness of SRS-A in this preparation.

Prostaglandin $F_{2\alpha}$

In an experiment using five rats, intravenous injection of 50 μg of prostaglandin $F_{2}\alpha$ produced only a weak but significant ($P < 0.05$) bronchoconstriction (Table 1). Because of the lack of efficacy of prostaglandin $F_{2\alpha}$ no experiments with antagonists were performed.

Discussion

Comparison of the activity of mediators of anaphylaxis on rat, guinea-pig and human bronchial function shows that the rat responds in a manner both qualitatively and quantitatively differently from the other two species.

The major qualitative difference is in the reactivity to histamine. In man and the guinea-pig, histamine is a potent bronchoconstrictor substance (Curry, 1946; Herxheimer, 1951; Berry & Collier, 1964). In the rat, however, histamine in doses up to 1 mg intravenously failed to cause bronchospasm. Similar findings have been reported *in vivo* by Bhoola, Collier, Schachter & Shorley (1962) and *in vitro* by Brocklehurst (1958) although Foggie (1937) found weak constriction in isolated perfused lung with large doses of histamine. Lack of reactivity of the bronchial tree to histamine has also been observed in the cat (Maengwyn-Davies, 1968) and the sheep (Eyre, 1969).

In the rat, 5-HT was the most potent bronchoconstrictor substance examined, giving an ED_{50} of 6.4 μg. 5-HT is also spasmogenic in the guinea-pig where the bronchial tree appears to be more sensitive than that of the rat (Berry & Collier, 1964). In man, 5-HT, injected intravenously or given by aerosol, causes no bronchospasm in normal subjects (Brocklehurst, 1958) and only slight bronchospasm in asthmatics (Michelson, Holander & Lowell 1958).

The spasmogenic activity of 5-HT in the rat was antagonized by methysergide. In acute anaphylaxis, methysergide antagonizes bronchoconstriction by nearly 70% (Church *et al.*, 1972). The sensitivity of the rat lung to 5-HT and the blockade of 5-HT and antigen-induced bronchospasm by methysergide implicates this amine as the major mediator of rat anaphylactic bronchoconstriction.

The partial inhibition of 5-HT-induced bronchospasm by atropine is not seen in the guinea-pig (Holgate & Warner, 1960) and was not expected in the rat. Two possible explanations for this may be put forward. Firstly, that the inhibition by atropine was due to a non-specific antagonism of amines (Vane, 1957). Secondly, that the rat lung contains neurotropic or M receptors for 5 HT, similar to those found in guinea-pig ileum and calf tracheal muscle, which excite cholinergic neurones rather than exert a direct effect on smooth muscle (Gaddum & Picarelli, 1957; Offermeier & Ariens 1966)

Acetylcholine causes contraction of bronchial muscle in most species including guinea-pig, rat and man (Brocklehurst, 1958, Bhoola *et al.*, 1962). Its ED_{50} in the rat was 20 μg intravenously which shows that the guinea-pig is more sensitive than the rat to acetylcholine. Although atropine blocks acetylcholine-induced bronchospasm in the rat, its failure to block anaphylactic bronchoconstriction (Church *et al.*, 1972) suggests that acetylcholine is not a primary mediator of allergic bronchospasm in this species. Similarly, atropine does not block anaphylactic bronchoconstriction in the guinea-pig (Collier & James, 1967) and is relatively ineffective in relieving the symptoms of asthma (Altounyan, 1969).

The sensitivity of rat lung to bradykinin was lower than that of guinea-pig and man. In the rat the ED_{25} of bradykinin was 41 μg whilst in the guinea-pig severe bronchospasm may be induced with 0.25-4 μg of bradykinin (Bhoola *et al.*, 1962; Collier, James & Piper, 1968). Bradykinin-induced bronchoconstriction in the rat is similar to that of the guinea-pig (Collier, Holgate, Schachter & Shorley, 1960) in that it is slower than the response to 5-HT. Recovery, however, is faster in the rat. A similar action in both species is suggested by the finding that the lung does not readily return to its predosed state and that tachyphylaxis develops with repeated dosing.

In the guinea-pig, but not in the rat bradykinin-induced bronchospasm may be blocked by meclofenamate and indomethacin (Collier, *et al.*, 1968). Piper & Vane (1969) and Palmer, Piper & Vane (1973) postulate that these compounds act in the guinea-pig by inhibiting the release of rabbit aorta contracting substance (RCS) by bradykinin. The failure of meclofenamate and indomethacin to block bradykinin-induced bronchospasm in the rat and the finding of Piper (personal communication) that histamine-like, SRS-A-like and prostaglandin-like, but no RCS-like activity, could be demonstrated in the perfusate from rat lung during anaphylaxis, suggest that RCS may not be involved in anaphylactic bronchoconstriction in the rat. In man, bradykinin aerosol induces a mild bronchospasm only in asthmatic patients, which is not inhibited by anti-inflammatory analgesic compounds (Streseman, 1963). In these respects man resembles the rat more closely than the guinea-pig.

SRS-A and prostaglandin $F_{2\alpha}$ cause bronchoconstriction in the guinea-pig and man (Herxheimer & Stresemann, 1963; Berry & Collier, 1964; Sweatman & Collier, 1968; James. 1969; Mathé, Hedqvist, Holmgren & Svanborg, 1973). In the rat, guinea-pig SRS-A, pharmacologically indistinguishable from rat SRS-A (Orange, Valentine & Austen, 1968), and prostaglandin $F_{2\alpha}$ both caused only slight constriction at unphysiologically high doses. Thus, the rat appears to differ from the guinea-pig and man in its sensitivity to these mediators.

Mediators of anaphylaxis release sympathomimetic amines (Feldberg & Lewis, 1964; Staszewska-Barczak & Vane, 1965. 1967; Piper, Collier & Vane, 1967) and blockade of β-adrenoceptors potentiated anaphylaxis and the effects of mediators, especially bradykinin (Collier, James & Piper, 1965, Collier & James, 1967). β-blockade also exacerbates human asthma (McNeill, 1964). Responses of the rat lung to bradykinin and SRS-A were potentiated by β-blockade less than were those of the guinea-pig (Collier *et al.*, 1965) whilst responses to 5-HT, acetylcholine and antigen (Church *et al.*, 1965) were not potentiated. This suggests that sympathomimetic amines are less effective on rat lung than on guinea-pig lung.

Although guinea-pig anaphylaxis is not inhibited by corticosteroids (Hicks, 1969), the activity of these drugs against rat anaphylactic bronchoconstriction (Church & James 1969, Church *et al.*, 1972) accords with their efficacy against human bronchial asthma. Rat bronchospasm induced by 5-HT or acetylcholine was not inhibited by dexamethasone, although Blythe (personal communication) found inhibition of the 5-HT response 24 h after intraperitoneal administration of 5 mg/kg of betamethasone. The inhibition of bradykinin bronchospasm by dexamethasone may be due to either a direct modulation of the systemic actions of kinins (Suddick 1966) or a potentiation by steroid of the actions of catecholamines (Logsdon, Middleton & Coffey, 1972) released by bradykinin. From this evidence it would seem unlikely that the anti-anaphylactic action of dexamethasone is due to its inhibition of the activity of mediators.

Human asthmatics are more sensitive to inhaled histmaine and prostaglandin $F_{2\alpha}$ than are non-asthmatics (Curry, 1946; Mathé *et al.*, 1973). In rats, sensitization increases systemic toxicity to histamine (Keller & Beeger, 1971). In my experiments, however, sensitization did not increase the sensitivity of rat lung to 5-HT, acetylcholine, histamine or bradykinin.

Rat anaphylactic bronchoconstriction resembles human asthma more closely than does guinea-pig anaphylaxis in its immunological triggering and its responsiveness to drugs that alleviate human asthma, namely disodium cromoglycate and corticosteroids (Church *et al.*, 1972). However, the qualitative differences in the responses to histamine and 5-HT and the quantitative differences in the responses to bradykinin, SRS-A and prostaglandin $F_{2}\alpha$ indicate that the reactivity of rat lung to mediators of anaphylaxis does not resemble that of human lung as closely as does that of the guinea-pig.

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(Received June 6, 1975
Revised July 21, 1975.)

Br. J. Pharmac. (1975), 55, 431-437

TOLERANCE TO MORPHINE-INDUCED CALCIUM DEPLETION IN REGIONAL BRAIN AREAS: CHARACTERIZATION WITH RESERPINE AND PROTEIN SYNTHESIS INHIBITORS

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1 Administration of a single dose of morphine sulphate (25 mg/kg) induces tolerance to calcium depletion lasting seven days.

2 There are no apparent changes in calcium content in any of eight discrete brain regions throughout this seven day period.

3 Pretreatment with reserpine (5 mg/kg) did not alter the ability of morphine to induce tolerance. Reserpine alone produced no tolerance to its own calcium depleting action.

4 Cycloheximide (500 μg/kg) but not chloramphenicol (200 mg/kg) effectively prevented development of tolerance.

5 It is concluded that the induction of tolerance to calcium depletion seen after morphine may involve changes in various proteins in membranes of synaptic origin.

Introduction

Requirement for calcium in membrane stabilization and function of neuronal systems is well established (Katz & Miledi, 1965; Triggle, 1972), particularly in excitation-coupling mechanisms (Somlyo & Somlyo, 1968, Douglas, 1968), and in the regulation of hormone-receptor interactions (Rasmussen, Goodman & Tenenhouse, 1972), but it has been only briefly studied in relation to mechanism of action of opiates. Kakunaga, Haneto & Hano (1966) reported reversal of morphine analgesia by calcium ions. Morphine has been found to alter calcium binding to phospholipids and gangliosides *in vitro* (Mule, 1969, Greenberg, Diecke & Long, 1972) and opiate ligand binding to isolated membrane fractions has been reduced by the addition of physiological concentrations of calcium (Pert & Snyder, 1973a,b; Hitzeman, Hitzeman & Loh, 1974).

Recent studies in this laboratory have been concerned with the *in vivo* effects of morphine on regional brain calcium (Ross, Medina & Cardenas, 1974). Morphine has been shown to decrease calcium in regional brain areas of the rat in a uniform fashion (Cardenas & Ross, 1975). This depletion was antagonized by naloxone, mimicked by levorphanol, but not by dextrophan. By the use of two non-analgesic central nervous system (CNS) depressants, reserpine and pentobarbitone, to characterize the morphine effect, evidence for more than one calcium pool in the CNS was obtained.

To establish an effect as directly related to opiate action, criteria such as those outlined above must be satisfied. In addition, a major criterion fulfilled in previous investigations has been the development of tolerance to the particular parameter being examined. In continuing an investigation into the role of calcium in the actions of morphine on the CNS, this study demonstrates the development of tolerance to the morphine-induced calcium depletion. Further, this tolerance is characterized by a calcium and amine depleting agent, reserpine, and protein synthesis inhibitor, cycloheximide. The results suggest that synthesis of membrane protein may be involved in the rapid induction of tolerance to the calcium depleting effect.

Methods

Preparation of tissues

Male Sprague-Dawley rats weighing 175-250 g were used in all experiments. Tissues were prepared from animals receiving saline (0.9% w/v NaCl solution) or the appropriate drug in saline solution according to methods previously described by Cardenas & Ross (1975). Tissues were prepared for calcium analysis by removing samples of eight anatomically discrete brain regions as outlined by Glowinski & Iversen (1966). The tissues were prepared from animals receiving saline or the appropriate drug in the equivalent volumes. Animals were killed at stated times; the brain was rapidly removed and rinsed in ice cold saline. Suitable amounts of tissue (10-25 mg) were removed and placed in preweighed disposable test tubes (Corning, diSPo, 16 x 100 mm culture tubes) together with 200 μl of concentrated HNO_3 (analytical grade) and the entire contents evaporated to a dry white ash over a hot plate. Any incomplete ashing as evidenced by dark brown flecks was treated with a second 200 μl volume of acid and the ashing procedure repeated. The residue was allowed to cool for 15 min then resuspended in a solution of 0.5 ml of 0.1 N HCl + 4.5 ml of 1% lanthanum (as the oxide in 0.1 N HCl). This solution was then read for calcium at the appropriate instrument settings.

Analysis of calcium

The concentration of calcium in regional areas of the rat brain was determined with the aid of a Perkin-Elmer atomic absorption spectrophotometer Model 403. The wavelength was set at 212 nm for calcium with a slit setting of 4, with the remainder of the instrument pre-adjusted in accord with the Perkin-Elmer Analytical Manual, 1973. A combination calcium-magnesium-zinc lamp was used with a current of 20 milliamperes. External standards were prepared by first diluting the calcium stock solution with 0.1 N HCl. Appropriate working standards were then prepared by further dilution in 1% lanthanum (as the oxide) in 0.01 N HCl. Lanthanum is included to prevent interference from high concentrations of phosphate normally found in brain tissue (Hanig & Aprison, 1967).

Materials

Drugs used in this study and their sources were as follows: morphine sulphate (Eli Lilly Laboratories, Indianapolis, Indiana), reserpine (Serpasil Brand, Ciba Geigy, Summit, New Jersey), cycloheximide (Sigma Chemical Corporation, St. Louis, New Jersey) and chloramphenicol (Chloramycetin, Park Davis & Company, Detroit, Michigan). Lanthanum and calcium standards were obtained from Research Chemical Corporation, Sun Valley, California, and Fisher Scientific Company, Fairlawn, New Jersey, respectively.

Statistics

Group and paired comparisons were made using Student's *t*-test as previously outlined by Snedecor & Cochran (1969).

Results

Time course of tolerance to a single dose of morphine

Our previous studies have demonstrated that calcium content in brain regions is maximally depressed 30 min after administration of morphine (Cardenas & Ross, 1975). After a single dose of morphine the average depletion throughout eight brain regions is approximately 35%. Calcium values returned to control levels within the first 24 hours. No changes were observed from this point throughout the next seven days when samples were studied at three and seven days.

To satisfy the criteria of tolerance to morphine as a reduced responsiveness to its repeated administration, the experiments outlined in Table 1 were performed. Each of three groups of animals received an induction dose of morphine (25 mg/kg) at zero time, and subsequently, another 25 mg/kg, 30 min before they were killed on days 1, 3 or 7. It can be concluded that, after a single induction dose, a second dose of morphine has no significant effect on brain calcium at days 1 and 3 since the regional values are not significantly different from the corresponding ones in rats treated with saline alone (Table 1) but are significantly different from those in rats given only one dose of morphine for 30 min (see footnote to Table 1). In contrast, calcium levels on day 7 in animals receiving an induction dose of morphine are lower than at days 1 or 3 although they are not decreased by as much as has been shown previously to occur 30 min after the administration of a single dose of morphine (Cardenas & Ross, 1975).

Effects of reserpine on calcium content

Reserpine was employed in an attempt to define more closely possible relationships between catecholamines and tolerance to the calcium-depleting effect of morphine. Previous studies (Cardenas & Ross, 1975) have demonstrated that values of the calcium content are reduced 2 h after the administration of reserpine (5 mg/kg) but return to normal at 24 hours.

Three groups of animals were used. One group received saline alone (Table 2, column 1). The second group received reserpine (5 mg/kg) followed 24 h later by one dose of morphine (25 mg/kg); the animals were killed 30 min after injection of morphine It can be seen (Table 2, column 2) that prior treatment with reserpine had no significant effect on the ability of morphine to cause a decrease in calcium content in these animals. The third group was used to evaluate the ability of reserpine to induce tolerance to its own calcium-depleting action. When reserpine was given as an induction dose of 5 mg/kg, followed after 24 h by a second dose (5 mg/kg), the calcium values were depleted (at 2 h) by the second dose. A comparison of these values may be made with the values of calcium 2 h after a single treatment with reserpine (5 mg/kg) (see footnote, Table 2), there is no significant difference.

Effects of reserpine on development of tolerance

Reserpine was also evaluated for its ability to alter tolerance. Table 3 depicts the effects of reserpine on tolerance to calcium depletion in eight brain regions. Reserpine was administered as a 2 h pretreatment followed by the tolerance regimen as

Table 1 Acute tolerance to calcium depleting effect of a single dose of morphine* in different brain regions of the rat

	Tissue calcium, μg/g wet wt. (mean ± s.e. mean)†			
Brain region	*Day 1*	*Day 3*	*Day 7*	*Saline-treated*
Hypothalamus	53.2 ± 0.5	57.0 ± 5.0	54.3 ± 4.7	55.8 ± 2.0
Hippocampus	55.3 ± 2.9	60.6 ± 3.3	44.6 ± 6.7‡	59.0 ± 2.1
Corpus striatum	57.2 ± 1.3	51.6 ± 1.2	48.6 ± 5.4	56.6 ± 1.9
Cortex	56.8 ± 1.4	57.4 ± 1.8	54.3 ± 3.8	52.1 ± 0.7
Cerebellum	54.2 ± 2.2	47.3 ± 4.6	39.3 ± 1.2‡	54.8 ± 0.8
Medulla pons	52.2 ± 4.3	46.0 ± 3.2	43.0 ± 3.7‡	54.8 ± 0.9
Midbrain	59.0 ± 4.6	54.0 ± 3.2	53.6 ± 3.7	56.0 ± 0.9
Thalamus	48.0 ± 2.1	51.0 ± 1.8	42.0 ± 3.5‡	54.1 ± 1.1

All values expressed are the mean of separate determinations from 8-12 animals.

* Morphine sulphate tolerance regimen was performed as follows: morphine (25 mg/kg i.p.) was given at zero time. Animals received a second dose 30 min before they were killed 1, 3 and 7 days later, animals were killed 30 min after the second morphine dose (25 mg/kg). Acute treatment with morphine (25 mg/kg) (30 min) has been previously shown to cause an average depletion of 35% throughout eight regions (Cardenas & Ross, 1975).

† Values for day 1 and day 3 are not significantly different from saline-treated rats.

‡ Values for day 7 represent transition from tolerance to non-tolerance state and are significant at $P < 0.01$.

Table 2 Effects of reserpine on morphine depletion of calcium in different regions of rat brain

	Tissue calcium, μg/g wet wt. (mean ± s.e. mean)		
Brain regions	*Control*	*Reserpine**	*Reserpine†*
Hypothalamus	55.8 ± 2.0	37.5 ± 4.1	40.8 ± 3.0
Hippocampus	59.0 ± 2.1	30.3 ± 2.5	37.5 ± 2.0
Corpus striatum	56.6 ± 1.9	33.3 ± 1.4	40.3 ± 2.5
Cortex	52.1 ± 0.7	33.5 ± 3.1	40.6 ± 0.9
Cerebellum	54.8 ± 0.8	38.8 ± 2.6	34.8 ± 2.8
Medulla pons	54.8 ± 0.8	39.4 ± 3.3	36.2 ± 2.2
Midbrain	56.0 ± 0.9	34.5 ± 3.6	41.2 ± 2.6
Thalamus	54.1 ± 1.1	37.2 ± 2.2	38.7 ± 2.1

Values are mean of separate determinations from 10-12 animals.

* Reserpine (5 mg/kg) given 24 h before morphine (25 mg/kg). Animals killed 30 min after morphine. All values significantly different from controls ($P = 0.001$). Animals given reserpine (5 mg/kg) followed 24 h later by saline did not have values significantly different from those of saline controls

† Tolerance to reserpine was evaluated by giving reserpine (5 mg/kg) at zero time followed by same dose 24 h later. Animals were killed 2 h after the second dose and calcium determined. All values are significantly different from controls. It has previously been shown that reserpine (5 mg/kg) causes an average calcium depletion of 33-36% throughout eight brain areas at 2 h (Cardenas & Ross, 1975).

outlined in Table 1. Only small decreases in brain calcium content were observed for day 1, 3 or 7 after reserpine pretreatment. The levels are not significantly different over all from those listed in Table 1 for days 1, 3 and 7 respectively.

Effects of protein synthesis inhibition on calcium content and development of tolerance

Cycloheximide (500 µg/kg, i.p.) or chloramphenicol (200 mg/kg, i.p.) were administered in a single dose to three groups of rats. One hour later, the tolerance schedule of injection of morphine was started as outlined in Table 1; Table 4 illustrates the results of these experiments. Calcium values after cycloheximide were significantly depleted at all three days compared to controls receiving appropriate solutions of saline instead of morphine However, chloramphenicol proved ineffective in altering the development of tolerance at day 1, 3 and 7. Both cycloheximide and chloramphenicol alone produced no changes in control values for calcium content. Cycloheximide did not alter the ED_{50} for calcium-depletion by morphine (Ross, unpublished

Table 3 Effects of reserpine* on development of tolerance to calcium-depleting effect of morphine

	Tissue calcium, µg/wet wt. (mean ± s.e. mean)		
Brain region	*Day 1*	*Day 3*	*Day 7*
Hypothalamus	53.6 ± 1.4	51.1 ± 2.7	42.6 ± 3 9
Hippocampus	51 2 ± 3.3	55.2 ± 1.7	51 6 ± 2.0
Corpus striatum	49.3 ± 1.2	52 1 ± 2.4	46.3 ± 4.4
Cortex	49.3 ± 1.2	52 1 ± 2.4	46.3 ± 4.4
Cerebellum	48.5 ± 4.1	46.8 ± 0.8	52.3 ± 3.0
Medulla pons	57.3 ± 2.7	45.7 ± 4.6	55.3 ± 2.3
Midbrain	46.6 ± 2.0	55.6 ± 1.6	56 0 ± 3 0
Thalamus	47.3 ± 5.6	54.1 ± 1.4	48.0 ± 4.0

Values represent mean of 8-10 animals.

* Reserpine (5 mg/kg) was administered as 2 h pretreatment followed by the morphine tolerance regimen outlined in Table 1. It has previously been demonstrated that reserpine alone (5 mg/kg) produced an average depletion of 33-36% in eight discrete brain regions (Cardenas & Ross, 1975).

Day 1, 3 or 7 significantly different from reserpine controls.

Table 4 Effects of cycloheximide* (Cyclo) and chloramphenicol† (Chlor) on development of tolerance to morphine in regional brain areas

	Tissue calcium, µg/g wet wt. (mean ± s.e. mean)						
		Day 1		*Day 3*		*Day 7*	
Brain region	*Control*	*Cyclo*	*Chlor*	*Cyclo*	*Chlor*	*Cyclo*	*Chlor*
Hypothalamus	55.8 ± 2.0	32.9 ± 2.9	55.9 ± 2.0	32.5 ± 2.6	54.6 ± 0.9	26.6 ± 0.3	51.7 ± 0.8
Hippocampus	59.0 ± 2.1	36.5 ± 4.2	57.0 ± 1.4	34.5 ± 2.5	55.3 ± 1.4	30.3 ± 1.8	52.5 ± 2.0
Corpus striatum	56.6 ± 1.9	34.0 ± 2.7	53.8 ± 2.1	34.7 ± 2.9	56.0 ± 1.0	25.6 ± 3.5	53.6 ± 0.8
Cortex	52.1 ± 0.7	30.7 ± 4.1	57.6 ± 3.6	35.7 ± 4.5	54.0 ± 1.6	32.3 ± 2.9	51.3 ± 1.1
Cerebellum	54.8 ± 0.8	31.6 ± 3.7	56.0 ± 2 8	31.0 ± 2.5	53.3 ± 1.6	26.5 ± 2 4	53.7 ± 0.8
Medulla pons	54.6 ± 1.5	33.3 ± 1.8	56.4 ± 1.1	31.3 ± 5.0	52.0 ± 0.5	33.3 ± 6.6	52 2 ± 2.3
Midbrain	56.0 ± 0.9	31.0 ± 0.9	57.0 ± 2.0	31.5 ± 2.6	56.2 ± 1.8	23.3 ± 2.6	50.5 ± 2.0

Values represent mean of separate determinations from 6-8 animals.

* Cycloheximide (500 µg/kg) or (†) chloramphenicol (200 mg/kg) administered 1 h before start of tolerance regimen for morphine.

No significant differences for any of the three days or any of the individual brain regions when compared to controls. Corpus striatum, cerebellum and thalamus were significantly lower after cycloheximide on the seventh day ($P = 0.01$).

observation) an observation which has also been made by Tulunay & Takemori (1974).

Discussion

Administration of a single dose of morphine sulphate (25 mg/kg) induces tolerance to depletion of brain calcium for periods up to seven days. Throughout this period, there were no significant changes in any of the regional brain areas. Acute tolerance to a second dose of morphine persisted three days after the first exposure of the animal to the drug. By the seventh day, however, tolerance to the second dose of morphine was beginning to decline. Tolerance to single doses of opiates over short time periods has been previously reported. For example, Smith, Karmin & Gavitt (1966) administered levorphanol in single doses to mice and found tolerance to the cataractogenic effect of a second dose. This dose was administered within a period of five days. Cox, Ginsburg & Osman (1968) reported tolerance to analgesia as early as 4 h after the beginning of 5-10 mg kg^{-1} h^{-1} constant infusion.

Many attempts have been made to link changes in tissue levels and turnover rates of certain neurotransmitters with the development of tolerance to various actions of opiates. Theoretical possibilities for tolerance mechanisms involving cellular adaptation have also been based on alteration of levels of and sensitivity to various neurochemical mediators. Reserpine, which has been previously shown to alter brain calcium levels (Raduco-Thomas, 1971; Cardenas & Ross, 1975), did little to prevent the development of tolerance to the morphine-induced depletion of calcium. This lack of effect of reserpine in preventing tolerance may not be surprising. It has been previously demonstrated that at least two pools of calcium exist, one morphine-sensitive and protected by naloxone, the other reserpine-sensitive but not protected by naloxone (Ross *et al.*, 1974; Cardenas & Ross, 1975). The calcium depleted by reserpine would conceivably leave the morphine-sensitive pool intact. Supporting this idea of distinct pools of calcium is the observation that while reserpine causes calcium depletion, it does not produce tolerance to its own depleting effect (Table 2) suggesting that some particular structural requirement of the membrane may be necessary for activating tolerance to calcium depletion. Secondly, treatment with reserpine 24 h before a single dose of morphine did not alter the ability of morphine to deplete calcium (Table 2), nor the development of tolerance (Table 3). These observations would also suggest that optimum endogenous levels of biogenic amines may not be required for morphine to induce depletion of calcium.

In contrast to the lack of effect of reserpine on the development of tolerance, pretreatment with the protein synthesis inhibitor cycloheximide produces a significant blockade of tolerance development for periods lasting up to seven days (Table 4). The mechanism of action for cycloheximide has been reported as the direct prevention of ribosomal movement along the m-RNA chains (Wettstein, Noll & Penman, 1964; Colombo, Felicetti & Boglioni, 1965). Therefore, it might be reasoned that the induction of tolerance to the depletion of calcium may in some way be connected to the translational aspect of protein synthesis. In support of this reasoning, Datta & Antopol (1973) have demonstrated in mice that chronic opiate treatment produces a decrease in aminocyl synthetase activity that is both dose and time-dependent.

In contrast to the effects seen after cycloheximide treatment, chloramphenicol (200 mg/kg) had no effect on development of tolerance to calcium depletion when administered as a single dose prior to initiation of tolerance. Previous investigations have shown that cycloheximide as well as other inhibitors of protein synthesis effectively abolish tolerance initiated by opiate drugs (Cohen, Keats, Krivoy & Ungar, 1965; Loh, Shen & Way, 1969; Cox & Osman, 1970; Feinberg & Cochin, 1972). Our data confirm these observations with cycloheximide and also support the lack of effects of chloramphenicol on tolerance development previously reported by Cox & Osman (1970). The inability of chloramphenicol to suppress the development of tolerance significantly may be explained in the following manner. Although the drug is reported to alter mammalian protein synthesis (Wheeldon & Lehninger, 1966), it has been previously demonstrated that chlroamphenicol does not alter protein synthesis in cerebral tissue in doses five time greater than that required to alter liver synthesis activity (Gordon & Deanin, 1968). The lack of effect may also be related to its action at mitochondrial protein synthesis, rather than protein synthesis of synaptic membrane origin, as suggested by Morgan & Austin (1968). In support of this idea, Barondes (1974) has recently reported studies indicating about 80% of protein synthesis by synaptosome fractions is inhibited by cycloheximide while about 20% is sensitive to chloramphenicol and is thought to be of mitochondrial origin.

Data presented in this paper demonstrate that tolerance to morphine may be induced by a single dose and persist for periods of up to seven days Cycloheximide acted effectively to prevent induction of tolerance while chloramphenicol was without activity. Reserpine, previously shown to interrupt analgesia tolerance, was also without effect. Based on the presumed site of action of cycloheximide, it is suggested that rapid changes in protein synthesis may take place within the synaptic membrane. Tolerance to morphine may involve rapid changes in the calcium-protein associations of the synaptic membranes.

This research was supported in part by NIDA-01168 and Pharmaceutical Manufacturers Association Research Grant.

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(Received June 18 1975.
Revised August 4 1975.)

Br. J. Pharmac. (1975), 55, 439-445

ADENOSINE AND DIPYRIDAMOLE ACTIONS AND INTERACTIONS ON ISOLATED CORONARY ARTERY STRIPS OF CATTLE

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1 The actions and interactions of adenosine and dipyridamole were investigated on isolated strips of coronary arteries of beef cattle. It was found that small diameter arteries (about 0.5-1.0 mm o.d.), raised to a moderate level of tone with potassium, responded with relaxation to low concentrations of adenosine

2 Dipyridamole, over a broad concentration range (6.0×10^{-8}-2.0×10^{-5}M), enhanced these responses, shifting the adenosine concentration-response curve (3.7×10^{-8}-1.1×10^{-4}M) considerably to the left. In contrast, inhibitory concentration-response curves to sodium nitrite and to noradrenaline were not materially altered by dipyridamole.

3 Studies of the uptake of [^{3}H]-adenosine revealed a rapid uptake of the nucleoside by coronary artery strips, which was inhibited by dipyridamole (6.0×10^{-8}-2.0×10^{-5}M); but this may not be sufficient to account fully for the observed sensitization.

4 It is concluded that the regulation of adenosine responses and the action of dipyridamole in the heart involve a more direct association with coronary vascular tissue than has been previously appreciated.

Introduction

The proposal has been made that adenosine, a product of adenine nucleotide utilization in cardiac tissue with vasodilator activity, is a regulator of coronary artery diameter (Berne, 1963). Major evidence in support of this hypothesis is the finding of elevated levels of adenosine in the heart, pericardial fluid and venous effluent during hypoxic conditions and, more recently, the observed enhancement of reactive hyperaemia in the presence of dipyridamole, an agent known to sensitize responses to exogenous adenosine (Miura, Tominaga & Hashimoto, 1967; Bittar & Pauly, 1971; Juhran, Voss, Dietmann & Schaumann, 1971; Parratt & Wadsworth, 1972a). Dipyridamole blocks the uptake of adenosine into red blood cells and into heart muscle as well, and the increased amounts of adenosine reaching the coronary vessels, in the presence of this inhibitor, are presumed to account for the magnified response. In addition, inhibition of adenosine inactivation has been invoked by some to explain, at least partially, the direct dilator effects of dipyridamole (Stafford, 1966; Nott, 1970).

The possibility that the uptake of adenosine into coronary vascular tissue itself, is a factor regulating responses to this agent has not been explored, nor has the possibility of a direct interaction between dipyridamole and adenosine at the level of the coronary vasculature been examined. In the present experiments, in which an *in vitro* preparation of bovine coronary artery strips was used to eliminate extrinsic factors, it was found that [^{3}H]-adenosine is taken up by vascular tissue and that dipyridamole inhibits this uptake and also directly and specifically sensitizes coronary artery responses to adenosine.

Methods

Hearts were removed within 10 min of slaughter of the cattle and immersed in oxygenated Krebs solution and transported to the laboratory. The total elapsed time was approximately 20 minutes. The left circumflex and descending coronary arteries or branches of the left descending coronary artery were dissected out, cleaned of visible fat and adherent tissue and cut into spiral strips of about 23 x 2.5 mm and 23 x 1.5 mm, respectively. The strips were suspended under 2 g tension (or in the case of the branch vessels 1 g tension) in 15 ml muscle chambers at 37°C containing Krebs-Henseleit solution of the following composition (mM): NaCl 115.3, KCl 4.6, $CaCl_2$ 1.8, $MgSO_4$ 1.1, $NaHCO_3$ 22 1, KH_2PO_4 1.1, glucose 7.8; to this medium disodium ethylenediamine tetraacetic acid (EDTA) (0.03 mM) was added to retard heavy metal catalyzed oxidation of catecholamines. A 60 min period was allowed for equilibration before eliciting drug responses. Isotonic contractions were recorded by means of frontal writing levers on a slowly moving kymograph drum (about 1.8 mm/min) with a lever magnification of 6.8-fold as described previously (Kalsner, 1974a).

All drug concentrations are expressed in terms of molarity. Potassium chloride, sodium nitrite, adenosine and dipyridamole were dissolved to the appropriate concentration in distilled, demineralized water. (−)-Noradrenaline was diluted to the desired concentration in 0.9% w/v NaCl solution containing 0.01 N HCl. Control and treated strips for each experimental protocol were taken from the same preparations. This was made necessary by the variation in the absolute response magnitudes to adenosine of strips from different preparations. Strips were used to obtain only one dose-response curve to a given agonist and then discarded.

[^{3}H]-adenosine uptake protocol

Adenosine [2,8-^{3}H] (30 mCi/μM) (New England Nuclear) was diluted to a stock concentration of 30.0 μCi/ml (1 μM) in water and stored frozen in 1 ml aliquots under nitrogen gas. Aliquots were thawed only once, immediately prior to use. For uptake studies strips of coronary artery were prepared as described above and pre-incubated individually at 37°C for 30 min in test tubes containing 10 ml Krebs solution. Aeration with 95% O_2 and 5% CO_2 was maintained throughout. The strips were then incubated for 10 min with [^{3}H]-adenosine (300 nCi/ml). The final concentration of [^{3}H]-adenosine was 1×10^{-8} M or 5×10^{-6} M; the latter achieved by dilution with non-radioactive adenosine. Dipyridamole when used, was added 15 min prior to incubation with the tritiated adenosine.

After incubation with [^{3}H]-adenosine for 10 min, the strips were blotted gently, weighed, chopped and placed in scintillation vials containing 2 ml of solubilizer (protosol, New England Nuclear) and 0.2 ml of distilled water to promote dissolution of the tissue. The vials were then maintained overnight in a water bath at 50°C to speed solubilization. Fifteen ml of scintillation solution (4 g of PPO and 50 mg of POPOP per litre of toluene) was added to each vial and the samples counted to a 2% error in a Beckman LS-150 system using automatic external standardization, to determine efficiency. Uptake of radioactivity is expressed as d/min per g of tissue after correction for extracellular space, assumed to be 0.35 ml/g tissue (Somlyo & Somlyo, 1968). The concentration of adenosine in the bathing medium was confirmed for each experiment by counting the radioactivity in 0.1 ml aliquots. The tissue to medium ratio was calculated as d/min per g of tissue (without extracellular space correction) divided by the d/min per ml of bathing medium.

Data analysis

Mean values of all data are shown with their standard errors and were compared by Student's *t*-test for unpaired data. Differences with *P* values of 0.05 or less were considered significant. Changes in response sensitivity at the ED_{10mm} level are expressed as the ratio of geometric mean values (Fleming, Westfall, De La Lande & Jellett, 1972). Each ED_{10mm} was converted to its log and the mean for each group recorded. The antilog of each mean log is presented as the geometric mean. Due to the lack of parallelism of certain of the dose-response curves, comparisons at other response levels would yield somewhat different values (Kalsner, 1947b), and this is evident from an examination of Figures 1 and 2.

Results

Responses of coronary arteries to adenosine

In preliminary experiments it was found that the large epicardial coronary arteries (o.d. 1-5 mm) did not respond to adenosine with any material relaxation when tested over a wide concentration range. This is in agreement with the finding of Schnaar & Sparks (1972) using dog vessels of similar dimensions. For this reason, smaller branch vessels of the left descending coronary artery (approximately 0.5-1.0 mm o.d.) were used for the present work. Artery strips first were constricted with potassium chloride (30 mM) to provide sufficient tone to obtain inhibitory dose-response curves to adenosine. After the contractions to potassium achieved a stable plateau level of response, e.g., a mean of 91.6 ± 7.6 mm in a typical set of 12 control strips (Figure 1a), cumulatively increasing concentrations of adenosine were added to the muscle chambers. Sufficient time was allowed between additions of the nucleoside for responses to plateau fully. The mean inhibitory concentration-response curves to adenosine (3.7×10^{-8}-1.1×10^{-4} M), obtained in four separate groups of control strips are presented

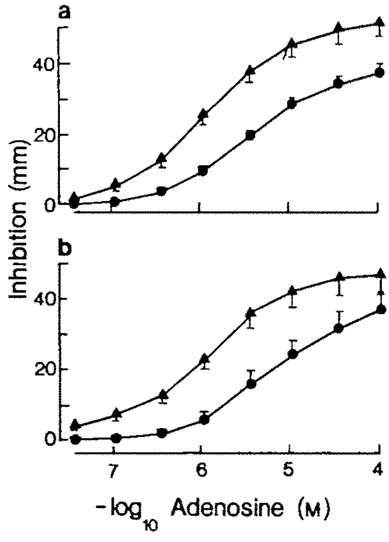

Figure 1 Effects of dipyridamole on the cumulative concentration-response curves to adenosine in coronary artery strips. Controls (●), dipyridamole-treated (▲). Concentrations of dipyridamole are 2.0×10^{-5} M and 6.0×10^{-6} M in (a) and (b). Number of control and treated values are 12 and 13 in (a) and 5 and 5 in (b). Mean values are shown; vertical lines show s.e. mean. Details are provided in text.

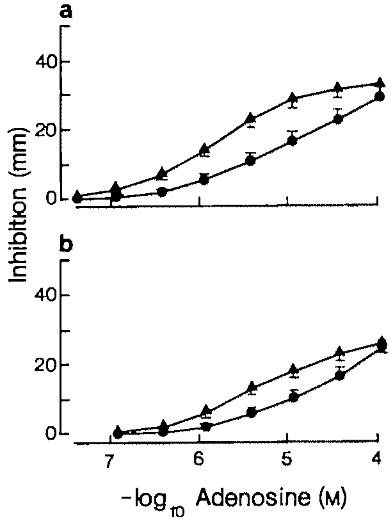

Figure 2 Effects of low concentrations of dipyridamole on the adenosine concentration-response curve. Controls (●), dipyridamole-treated (▲). Concentrations of dipyridamole are 6.0×10^{-7} and 6.0×10^{-8} M in (a) and (b). Number of control and treated values are 11 and 12 in (a) and 6 and 11 in (b). Mean values are shown; vertical lines show s.e. mean. Details are given in text.

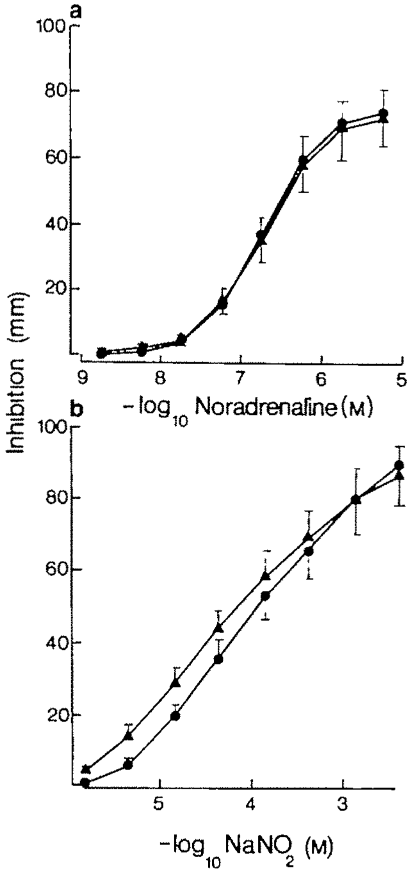

Figure 3 Effects of dipyridamole on the cumulative concentration-response curves to noradrenaline and sodium nitrite in coronary artery strips. Controls (●), dipyridamole-treated (▲) (2.0×10^{-5} M). Number of control and treated values are 11 and 13 in (a) and 11 and 11 in (b) Mean values are shown; vertical lines show s.e. mean. Details in text.

in Figures 1 and 2. The capacity of the strips to relax further is not usually exhausted after the response to adenosine 1.1×10^{-4} M is fully achieved, since sodium nitrite, a non-competitive inhibitor of muscle tone, additionally relaxed them to their pre-potassium baselines. However, concentrations of adenosine above 1.1×10^{-4} M were not routinely employed in the present experiments since an abrupt and apparently non-specific depression of tone was evident at the high concentration of 3.7×10^{-4} M.

Effects of dipyridamole on adenosine responses

To explore the effects of dipyridamole on the adenosine concentration-response curve, coronary artery strips taken from the same preparations as controls were contracted by potassium and exposed to dipyridamole (6.0×10^{-6} or 2.0×10^{-5} M) and 15 min later, without washout, responses to adenosine were obtained (Figure 1a and b). Dipyridamole, itself, in these high concentrations occasionally exerted a direct depressant effect on coronary artery tone but it was only a small percentage of the total potassium contraction amplitude; e.g., a mean inhibition of 6.1 ± 1.8% in 13 strips after dipyridamole (2.0×10^{-5} M). The adenosine concentration-response curves were shifted considerably to the left by both concentrations of dipyridamole (Figure 1a and b). The increase in sensitivity measured as the ratio of geometric means (control vs treated) at the ED_{10mm} response level was 9.8 and 7.2 after 6.0×10^{-6} and 2.0×10^{-5} M, respectively. These values did not differ significantly from each other.

In separate experiments again with control and treated strips taken from the same preparations to minimize variability, lower concentrations of dipyridamole (6.0×10^{-7} and 6.0×10^{-8} M) were also found to enhance significantly responses to adenosine (Figure 2a and b). The ratio of geometric means at the ED_{10mm} level (control/treated) was 6.0 and 3.7 respectively. In other experiments it was determined that a further 10-fold reduction in the concentration of dipyridamole, to 6.0×10^{-9} M, did not alter significantly the shape or position of the adenosine concentration-response curve when compared to matching control strips ($P > 0.2$).

To provide information on the specificity of the enhancing effects of dipyridamole, strips were contracted by potassium, as above, and complete inhibitory concentration-response curves to noradrenaline (1.8×10^{-9} to 6.0×10^{-6} M) and sodium nitrite (1.4×10^{-6} to 1.4×10^{-2} M) were obtained in the presence and absence of dipyridamole (2.0×10^{-5} M). Noradrenaline relaxes bovine coronary artery strips through β-adrenoceptor activation as described previously (Kalsner, 1974a). As indicated in Figure 3(a) and (b) responses to these agonists were not increased materially, even by this high concentration of dipyridamole, although a slight shift to the left of the lower portion of the $NaNO_2$ curve did occur. However, this shift was statistically significant only at the single agonist concentration of 4.3×10^{-6} M. The direct dilator effect of dipyridamole exerted on some preparations, and referred to above, may explain this slight enhancement, confined to the lower portion of the concentration-response curve. Since the catabolism of adenosine leads to the formation of inosine and hypoxanthine, the direct effects of these agents on coronary vascular tone was also studied. Over a wide concentration range (3.7×10^{-8} to 1.1×10^{-4} M and 7.4×10^{-8} to 2.2×10^{-4} M) neither inosine nor hypoxanthine inhibited potassium-induced tone.

Uptake of [^3H]-adenosine into coronary arteries

Direct evidence for the uptake of adenosine into coronary artery strips and its inhibition by dipyridamole was obtained by incubation of strips with [^{3}H]-adenosine at 1×10^{-8} and 5×10^{-6} M in the presence and absence of the sensitizing agent,

Table 1 Uptake of [^{3}H]-adenosine into coronary artery strips and the effects thereon of dipyridamole

	No. of strips	*Dipyridamole conc. (M)*	*Uptake (d/min × 10^{-3} per g)*	P value*	*% Inhibition of uptake*
[^{3}H]-Adenosine (1×10^{-8} M)	8	—	3146 ± 435	—	—
	6	6×10^{-9}	3390 ± 516	>0.5	—
	5	6×10^{-8}	1775 ± 210	<0.001	43.6
	7	6×10^{-7}	654 ± 85	<0.001	79.2
	7	6×10^{-6}	534 ± 23	<0.001	83.0
	4	2×10^{-5}	342 ± 16	<0 001	89.1
[^{3}H]-Adenosine (5×10^{-6} M)†	9	—	1048 ± 108	—	—
	10	6×10^{-7}	924 ± 97	>0.3	11.9
	13	6×10^{-6}	736 ± 87	<0.05	29.8
	9	2×10^{-5}	466 ± 122	<0 01	55 6

* Comparisons are with corresponding control group without dipyridamole treatment † Diluted with non-radioactive adenosine as detailed in methods section. Means are presented with their standard errors Strips were exposed to [^{3}H]-adenosine in the presence or absence of dipyridamole, and the accumulation of radioactivity after a 10 min incubation period is expressed after correction for extracellular space. Details are provided in text.

over a wide concentration range. With the lower concentration of adenosine, the vascular tissue rapidly accumulated tritium to reach a tissue/medium ratio of 4.3 after a 10 min incubation and this accumulation was strikingly inhibited by dipyridamole (6.0×10^{-8} M-2.0×10^{-5} M) (Table 1). However, in those strips exposed to a higher concentration of [^{3}H]-adenosine, one which produced a moderate response of the vascular strips, the tissue/medium ratio achieved was only 1.2 after 10 min and dipyridamole was much less effective in reducing uptake (Table 1). In other experiments it was found that strips of the left circumflex artery, although unresponsive to adenosine, also accumulated the nucleoside at the low concentration of 1×10^{-8} M, but did so less avidly than smaller, responsive, vessels. Control uptake gave a tissue/medium ratio of 2.3 and this was inhibited by dipyridamole as effectively as in responsive preparations of smaller arteries, a reduction of 69, 75 and 78% after treatment with 6×10^{-8}, 6×10^{-7} and 6×10^{-6} M of the antagonist respectively.

Discussion

Considerable evidence has been advanced to support the hypothesis that adenosine, an endogenously formed metabolite of adenine nucleotides in myocardial cells, functions as a regulating mechanism to adjust coronary vessel diameter, and hence coronary blood flow, to myocardial needs (Rubio, Berne & Katori, 1969; Berne, Rubio, Dobson & Curnish, 1971; Rubio, Berne & Dobson, 1973; Berne & Rubio, 1973). It has been proposed that adenosine is released continuously from myocardial cells into the interstitial fluid where it contacts the resistance vessels, in amounts directly relaxed to cellular metabolic rates (Scott, Daugherty, Dabney & Haddy, 1965; Katori & Berne, 1966; Rubio & Berne, 1969). Important impetus for this hypothesis has come from reports that dipyridamole, an inhibitor of adenosine uptake into erythrocytes and myocardial tissue (Koss, Beisenherz & Maerkisch, 1962; Gerlach & Deuticke, 1963; Bunag, Douglas, Imai & Berne, 1964; Pfleger, Voikmer & Kolassa, 1969; Kolassa, Pfleger & Rummel, 1970; Hokins, 1973), enhances vasodilator responses to exogenous adenosine in perfused heart preparations (Bretschneider, Frank, Bernard, Kochsiek & Scheler, 1959; Stafford, 1966; Moir & Downs, 1972; Sano, Sato & Hashimoto, 1972; Parratt & Wadsworth, 1972b) and the hyperaemic response to coronary artery occlusion, as well (Miura *et al.*, 1967; Bittar & Pauly, 1971, Juhran *et al.*, 1971; Parratt & Wadsworth, 1972a). However, this latter finding is not universally accepted (Kubler, Spieckermann & Bretschneider, 1970) and is interpreted by some to indicate a role for adenosine only in the more severe hypoxic stresses (Parrat & Wadsworth, 1972a; Moir & Downs, 1972).

The present experiments were undertaken to determine if coronary arterial tissue has the capacity to take up extracellular adenosine and to explore the possibility that adenosine and dipyridamole interact directly at the level of the coronary vasculature rather than solely indirectly by an action of the inhibitor at sites remote from the site of action of adenosine. For this reason, preparations of coronary artery tissue suspended *in vitro* in Krebs medium were utilized.

Dipyridamole, (6×10^{-8}-2×10^{-5} M) exerted a direct action on vascular tissue to enhance responses to adenosine and to shift the entire concentration-response curve to the left. This action was specific for adenosine. Responses to noradrenaline and to sodium nitrite were not materially increased by the highest concentration of dipyridamole employed (2×10^{-5} M), providing no support for a previously reported sensitization of noradrenaline dilator responses by this agent, in perfused dog hearts (Hashimoto & Sano, 1968). This confirms the report of Parrat & Wadsworth (1972b) that dipyridamole is ineffective in enhancing noradrenaline dilator responses in intact heart preparations. In addition it was found that coronary vascular tissue has a considerable avidity for adenosine as determined by its uptake of [^{3}H]-adenosine and this accumulation was reduced by dipyridamole. Hopkins (1973) has pointed out that, in cardiac tissue much of the accumulated adenosine is retained intracellularly in the form of nucleotides, and this may be the case with vascular tissue as well.

While no definite statements can be made regarding the relationship between degree of sensitization and percentage inhibition of uptake due to limitations in our knowledge of the topography of the cell surface and specifically the relative locations of receptors and uptake sites, the lack of a close correlation between them is apparent. Responses to adenosine (5×10^{-6} M) were greatly enhanced by dipyridamole (6×10^{-7}-2×10^{-5} M) but uptake was not correspondingly reduced. A 50% and 65% inhibition of uptake is required to explain a 2 and 3-fold sensitization (dose-ratio); and uptake must be reduced by 90% to account for a 10-fold sensitization. It seems likely, therefore, that at least a component of dipyridamole sensitization may involve an action on vascular cells unrelated to inhibition of an uptake mechanism. In this connection, Hopkins (1973) previously reported that the enhancing action of lidoflazine on the responses of guinea-pig atria to adenosine could not be accounted for simply by its known ability to block myocardial uptake of the nucleoside.

The specific sensitization of vascular responses coupled with the inhibition of adenosine uptake indicates that the action of dipyridamole and 'dipyridamole-like' compounds involves a more intimate association with coronary vascular tissue than has been previously appreciated. Berne has proposed that newly-formed adenosine leaves the heart muscle, enters the interstitial fluid, acts on resistance vessels and then has its action terminated by uptake into erythrocytes and to some extent into cardiac tissue, where it is either degraded to inosine or hypoxanthine, or incorporated into nucleotides via adenosine kinase (Berne, 1963). Dipyridamole would sensitize action by blocking this uptake thus diverting increased amounts of adenosine to the vascular receptors and maintaining them there for longer periods. The present findings warrant an alternative explanation.

The magnitude of the sensitization observed here indicates that effects on vascular cells probably contribute in a major way to the potentiation of adenosine responses observed in the intact heart. Parratt & Wadsworth (1972a, b) have reported that dipyridamole produces a 2-8 fold sensitization of adenosine dilator responses in the intact heart, and it has been shown here that an effect of this magnitude could involve, to a significant extent, an interaction at the level of vascular tissue, the immediate site of adenosine action in the heart.

It is important to note that, although the amount of vascular tissue in the heart is an insignificant proportion of its total bulk, this is not the critical factor in determining the *in vivo* implications of the present finding. If adenosine reaches vascular sites of action in sufficient concentrations to elicit relaxation then the effects of dipyridamole on response magnitude will be similar to those observed in the present experiments; regardless of the absolute amounts of adenosine involved. In addition, regulation of coronary dilator responses to adenosine by other endogenous influences *in vivo*, also can be envisaged to occur through alterations in its rate of uptake into vascular cells. The present data therefore provide a new aspect for consideration in the currently controversial picture of the role of adenosine in the dynamics of coronary blood flow regulation.

The author thanks Mr Mohamad Suleiman for excellent technical assistance. This investigation was supported by grants from the Ontario Heart Foundation and the Medical Research Council of Canada.

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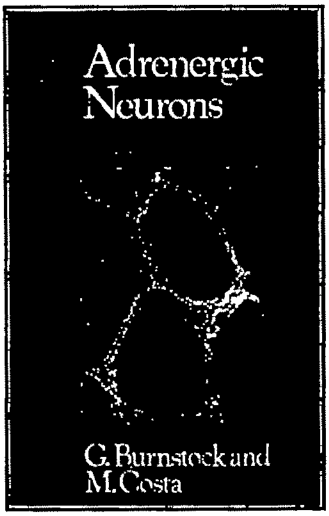

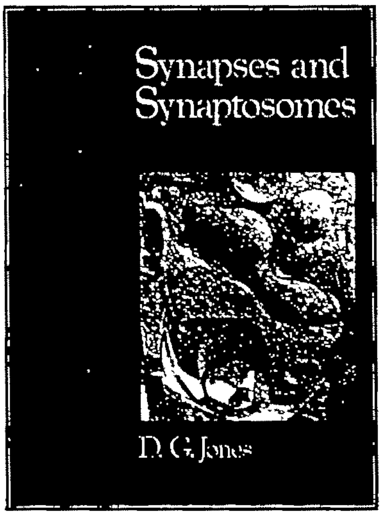

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THE ACTION OF ACETYLCHOLINE ANTAGONISTS ON AMINO ACID RESPONSES IN THE FROG SPINAL CORD *in vitro*

R.A. NICOLL[1]

Department of Physiology, School of Medicine, State University of New York at Buffalo,
Buffalo, New York 14214, U.S.A.

1 The isolated hemisected frog spinal cord has been used to study the action of acetylcholine antagonists on amino acid responses by means of sucrose gap recording.

2 Primary afferents and motoneurones were shown to contain few, if any, cholinoceptors, since acetylcholine and carbachol responses were essentially abolished when synaptic transmission was blocked with magnesium ions or when action potentials were blocked by tetrodotoxin.

3 Curare antagonized the γ-aminobutyric acid (GABA) and β-alanine depolarizations of primary afferents and the hyperpolarizing action of these amino acids on motoneurones. Nicotine also antagonized β-alanine depolarizations and to a small extent GABA depolarizations of primary afferents. These actions are similar to but weaker than those obtained previously with picrotoxin.

4 Atropine selectively antagonized β-alanine depolarizations of primary afferents and blocked β-alanine and glycine hyperpolarizations of motoneurones. GABA responses were entirely resistant to the action of atropine. These actions are similar to but 50 times weaker than those obtained previously with strychnine.

5 Dihydro-β-erythroidine, tetraethylammonium, and gallamine were entirely ineffective in antagonizing amino acid responses. Since these agents are known to block the dorsal root potential elicited by ventral root stimulation but have no effect on the amino acid responses of primary afferents, it is evident that a cholinergic step is involved in this pathway.

Introduction

It is generally believed that the convulsant properties of such drugs as strychnine, picrotoxin, and bicuculline arise, in part, from their ability to antagonize amino acid mediated synaptic inhibition in the central nervous system. The fact that a number of agents which antagonize acetylcholine, including curare (Eccles, 1946; Chang, 1953; Feldberg, Malcolm & Darian Smith, 1957; Phillis & Tebecis, 1967; Bhargava & Meldrum, 1969; Banerjee, Feldberg & Georgiev, 1970; Feldberg & Lotti, 1970), gallamine (Salmoiraghi & Steiner, 1963; Curtis, Ryall & Watkins, 1966; Halpern & Black, 1967; Pixner, 1967; Galindo, Krnjević & Schwartz, 1968; Grinnell, 1970), tetraethylammonium (Furukawa, 1955, Pixner, 1967; Grinnell, 1970) and atropine (Bonnet & Bremer, 1952; Phillis & Tebecis, 1967; Bhargava & Meldrum, 1969), also have potent excitatory effects on central neurones, raises the possibility that these agents might also interfere with amino acid mediated transmission. Indeed, the observation that curare blocks both cortical inhibition and the action of γ-aminobutyric acid (GABA) (Hill, Simmonds & Straughan, 1972) supports this notion. Furthermore, the observation that picrotoxin, bicuculline (DeGroat, 1970; Bowery & Brown, 1974) and strychnine (Alving, 1961; Phillis & York, 1967; Kehoe, 1972, Faber & Klee, 1974; Bowery & Brown, 1974) can antagonize responses to acetylcholine, at least in high concentrations, indicates that acetylcholine and neutral amino acid responses have features in common. Thus, the present study was undertaken to examine the effects of a number of acetylcholine antagonists on amino acid responses in the frog isolated spinal cord.

Methods

The dissection and sucrose gap recording method used for recording drug responses from frog (*Rana*

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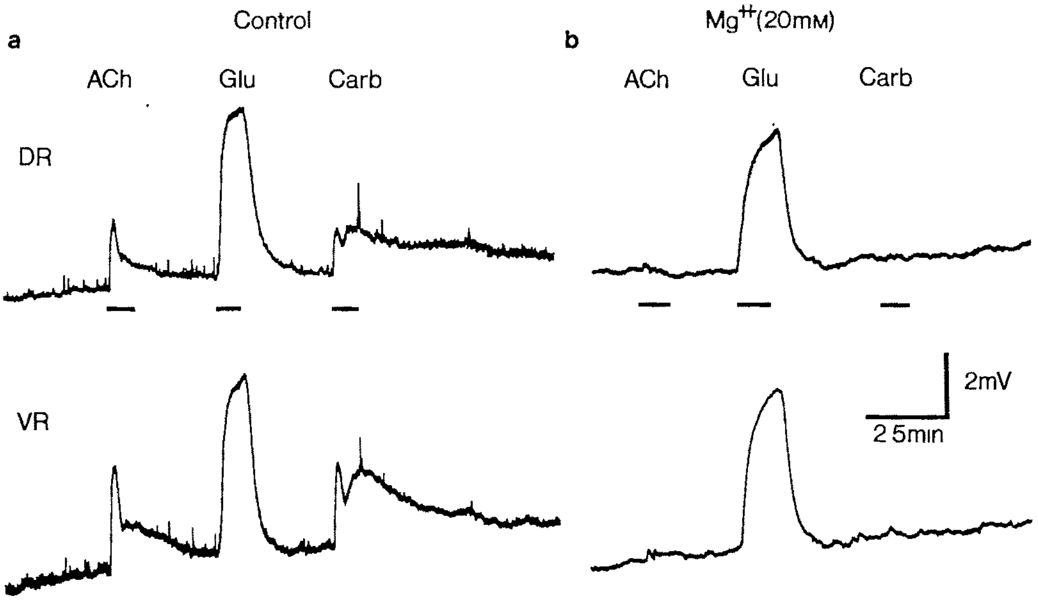

Figure 1 Effect of magnesium on cholinergic responses. (a) Upper record shows acetylcholine (ACh), carbachol (CCh) and glutamate (Glu) depolarizations on the primary afferents (DR) and lower record shows responses similarly recorded from motoneurones (VR). These responses were obtained in normal Ringer solution. Acetylcholine and carbachol are in a concentration of 10^{-2} M and glutamate 10^{-3} M. (b) Responses obtained after the addition of 20 mM magnesium sulphate to the Ringer solution. Note that the magnesium abolishes the responses to acetylcholine and carbachol.

pipiens) spinal roots has recently been described (Barker, Nicoll & Padjen, 1975a). In most preparations the responses from either two dorsal roots or two ventral roots were recorded simultaneously. On occasion, responses from one dorsal and one ventral root were recorded simultaneously (cf. Figure 1) (Barker *et al.,* 1975b). The drugs were made up in frog Ringer solution and the pH adjusted when necessary to 7.3 with HCl or NaOH. When the amino acids were tested during the application of an acetylcholine antagonist, the amino acids were prepared in the appropriate amount of antagonist, so that the antagonist would not be washed off during the amino acid application. To compare the sensitivity of various amino acid responses on the primary afferents to a particular antagonist, antagonist responses of equal size were usually used. For glutamate, GABA, and β-alanine the concentration range was from 2×10^{-4} M to 10^{-3} M. With this concentration range the responses for these amino acids were between 35 and 60% of their maximum response. Since the sensitivity of primary afferents to glycine is considerably less than to these other amino acids it was usually not possible to match this response without approaching the peak of the dose-response curve and using very high concentrations of glycine ($>5 \times 10^{-2}$ M). In such cases a compromise was reached whereby a glycine response of approximately 80% of the maximal response was used. However, to ensure that the resistance of the glycine response to a particular antagonist was authentic, responses ranging from 20 to 80% of the maximal response were also used. Since the size of the responses on the ventral root to the neutral amino acids was small and varied considerably in different preparations (Nicoll, Padjen & Barker, 1975), only a qualitative assessment was made as to whether a particular agent blocked the responses. Either magnesium sulphate (10 or 20 mM) or tetrodotoxin (50 μM) (Sigma) was added to the Ringer solution to block synaptic and interneuronal transmission, unless otherwise stated. The following drugs were used in the present study; β-alanine (Aldrich), γ-aminobutyric acid (Aldrich), atropine sulphate (Aldrich), dihydro-β-erythroidine (kindly donated by Merck, Sharp & Dohme), gallamine triethiodide (Davis & Geck), glutamate (K & K), glycine (Aldrich), nicotine (Sigma), and (+)-tubocurarine (Sigma).

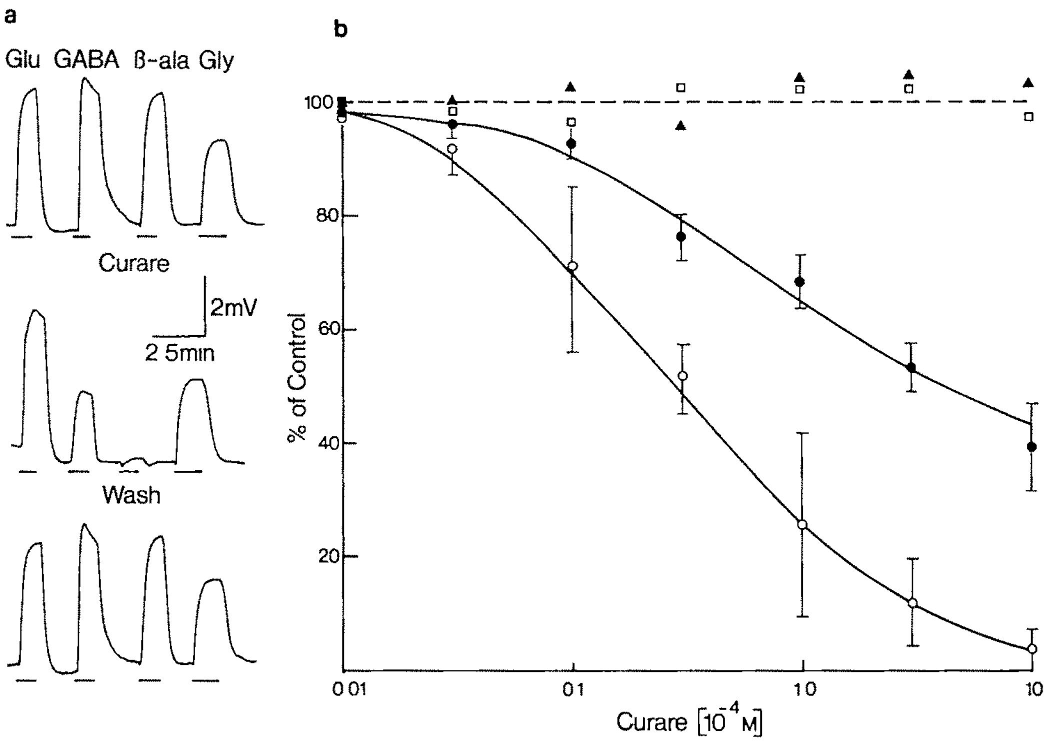

Figure 2 Curare antagonism of amino acid responses on primary afferents. (a) The effect of curare (10^{-3} M), applied for 95 min, on the depolarizing responses to glutamate (Glu, 3×10^{-4} M), γ-aminobutyric acid (GABA, 2×10^{-4} M), β-alanine (β-ala, 10^{-3} M) and glycine (Gly, 10^{-2} M). The bottom trace (Wash) was obtained 70 min after returning to a drug-free Ringer solution. In (b) the effects of increasing concentrations of curare on primary afferent depolarization produced by equi-effective concentrations of amino acids are plotted. The amplitude of the glycine response was 60-80% the size of the other amino acid responses (see methods section). The amplitude of the responses is expressed as a percentage of that obtained in the absence of curare. The responses at each concentration of curare were obtained after the responses had equilibrated (approximately 20 to 40 minutes) Each symbol represents the average of values obtained from at least three preparations and five dorsal roots. Responses to GABA (●); β-alanine (○); glutamate (▲) and glycine (□). The vertical bars give the standard deviation of the mean.

Results

Action of acetylcholine and carbachol on primary afferents and motoneurones

In studying the action of acetylcholine antagonists in the spinal cord it is important to know if the primary afferents and motoneurones have cholinoceptors, and if so, to characterize these pharmacologically. A depolarization of both the primary afferents and motoneurones was always produced when acetylcholine and carbachol (10^{-3}-10^{-2}M) were applied to the frog spinal cord with synaptic transmission intact. The acetylcholine and to a lesser extent the carbachol response faded quickly and often disappeared completely during the application (Figure 1a). Although a detailed analysis of the pharmacology of these responses has not been made, they were blocked entirely by atropine and were reduced by dihydro-β-erythroidine. These findings are in accord with previous investigations (Phillis & Tebecis, 1967; Koketsu, Karczmar & Kitamura, 1969). Since these responses were obtained in preparations exhibiting normal synaptic transmission, it is possible that they are mediated indirectly. Indeed, it was a consistent finding that the addition of magnesium or tetrodotoxin virtually abolished these responses, even when elicited with concentrations of 10^{-2} M (Figure 1b). In a few preparations magnesium ions or tetrodotoxin actually reversed the carbachol responses into small (<1 mV) hyperpolarizations (Nicoll, 1975). These results would suggest that the primary

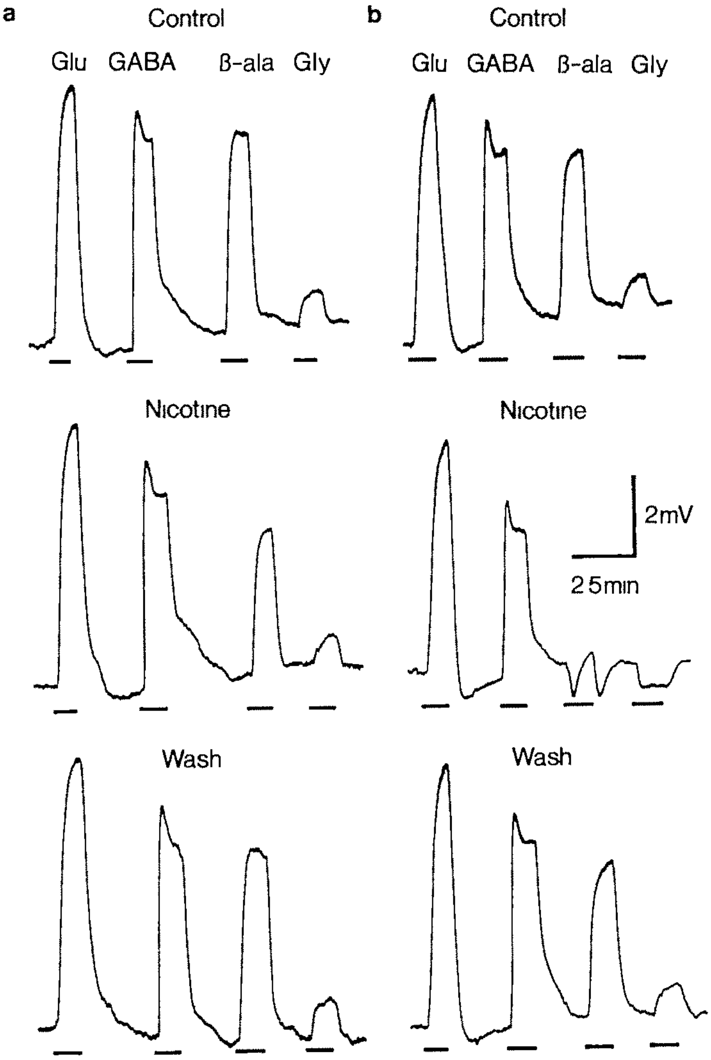

Figure 3 Nicotine antagonism of amino acid responses on primary afferents. In (a) 10^{-3} M nicotine, added 10 min prior to starting the middle record, reduced the β-alanine (β-ala) response but had little effect on the γ-aminobutyric acid (GABA) response. In (b) 4×10^{-3} M nicotine, added 13 min before starting the middle record, antagonized the GABA, β-alanine and glycine (Gly) depolarizations and revealed hyperpolarizing components to the β-alanine and glycine responses. The concentration of all the amino acids in (a) and (b) was 10^{-3} M except for glycine which was 2×10^{-3} M.

afferents and motoneurones have few, if any, receptors for acetylcholine.

Action of acetylcholine antagonists on amino acid depolarizations of primary afferent fibres

The acetylcholine antagonists tested did not have a common action on the amino acid responses. None of the agents had a direct effect on the membrane potential of primary afferents. Figure 2a shows the typical depolarization of the primary afferents by the amino acids, glutamate, γ-aminobutyric acid (GABA), β-alanine, and glycine. Curare was found to antagonize reversibly both the GABA and β-alanine response, but not the glutamate and glycine responses (Figure 2a). However, the β-alanine response appeared to be considerably more sensitive to curare than was the GABA

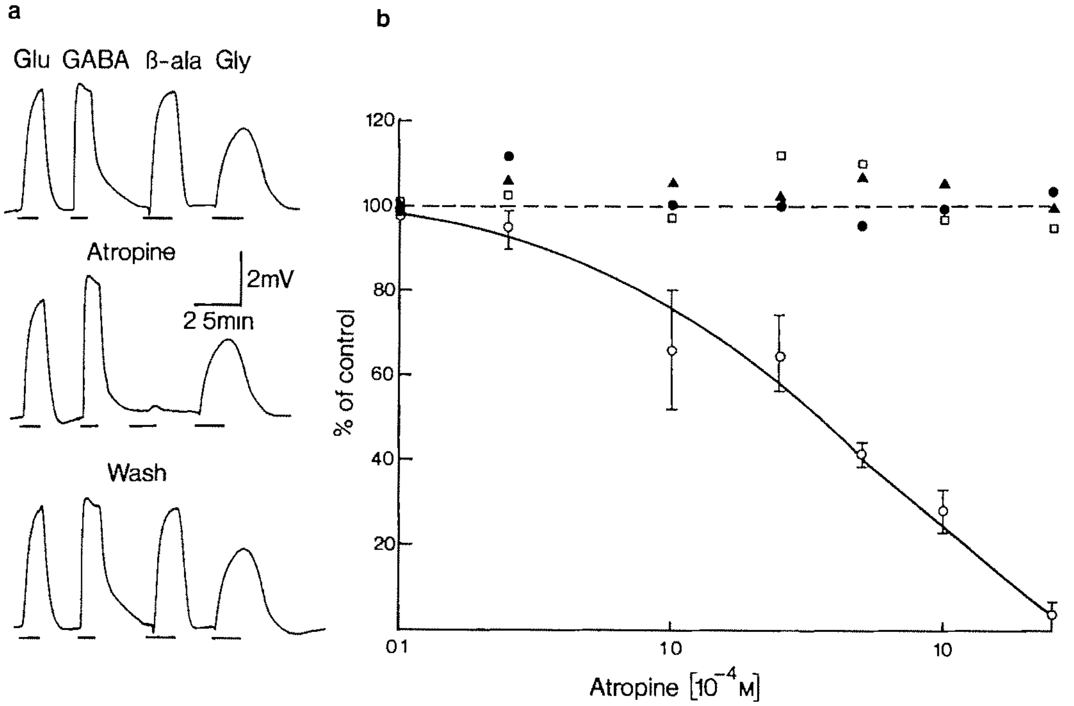

Figure 4 Atropine antagonism of amino acid responses on primary afferents. (a) The effect of atropine (2.5×10^{-3} M), applied for 75 min, on the depolarizing responses to glutamate (Glu, 4×10^{-4} M), γ-aminobutyric acid (GABA, 2×10^{-4} M), β-alanine (β-ala, 6.5×10^{-4} M), and glycine (Gly, 2×10^{-2} M). The bottom trace was obtained 90 min after returning to a drug-free Ringer solution. In (b) the effects of increasing concentrations of atropine on the primary afferent depolarization produced by equi-effective concentrations of amino acids are plotted. The amplitude of the glycine response was 60-80% the size of the other amino acid responses (see methods section). The amplitude of the responses is expressed as a percentage of that obtained in the absence of atropine. The response at each concentration of atropine was obtained after equilibration of the responses (approximately 15-30 minutes). Each symbol represents the average of values obtained in at least three preparations and four dorsal roots. Responses to GABA (●), β-alanine (○); glutamate (▲); and glycine (□). The vertical bars give the standard deviation of the mean.

response. To analyze the relative sensitivity of the amino acid responses to curare in more detail a wide range of antagonist concentrations were used in a number of preparations (Figure 2b). The threshold concentration for curare was approximately 3×10^{-6} M. At 10^{-3} M curare the β-alanine response was only 6.3% of control. The responses to both glutamate and glycine were unaltered by curare. Although not examined in detail, the response to taurine showed a similar sensitivity to curare as the β-alanine response. Thus the action of curare, although approximately 10 times weaker, is similar to that of picrotoxin (Nicoll & Barker, 1973; Barker *et al.*, 1975a).

Although nicotine (examined in six preparations) also antagonized amino acid responses, its action was very weak. With equimolar concentrations of amino acid and nicotine, nicotine reduced the β-alanine response, but had only a slight action against GABA (Figure 3a). With high concentrations (up to 4×10^{-3} M nicotine and 10^{-3} M amino acids) these effects became more pronounced (Figure 3b). However, these higher concentrations of nicotine also antagonized the glycine response and brought out a hyperpolarizing component to the glycine and β-alanine response (Figure 3b).

Unlike the action of curare and nicotine, the action of atropine was entirely selective against the β-alanine responses (Figure 4). GABA, glutamate and glycine responses were resistant to atropine, even in concentrations of 2.5×10^{-3} M. Figure 4b summarizes the effect of increasing concentrations of atropine on amino acid responses from a number of preparations. The antagonism of β-alanine response is detectable at approximately 2.5×10^{-5} M and becomes complete at approxi-

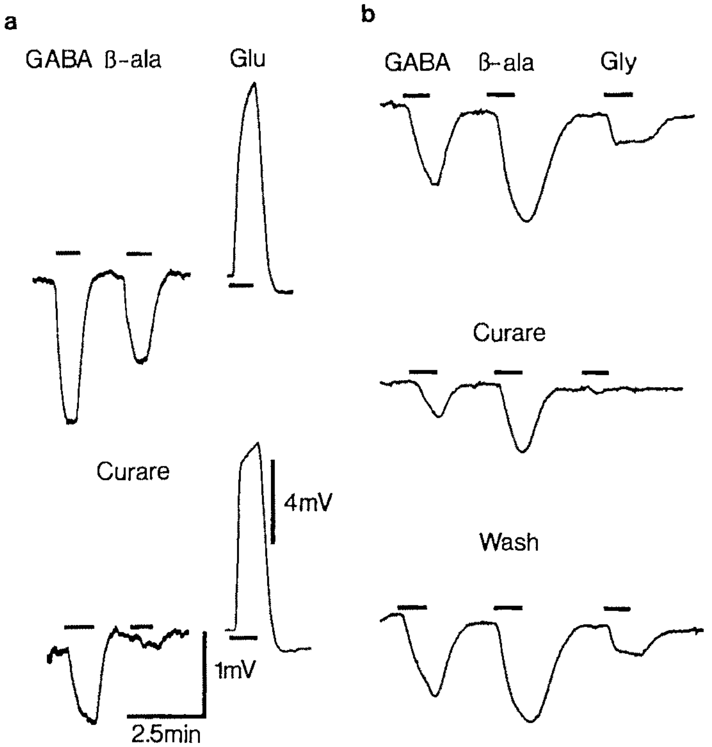

Figure 5 Curare antagonism of amino acid hyperpolarizing responses from motoneurones. (a) Antagonism of γ-aminobutyric acid (GABA) and β-alanine (β-ala) hyperpolarizations by curare, but not the glutamate depolarizing response The responses in curare (5×10^{-4} M) were obtained 10 min after addition of curare. The concentration of all the amino acids was 5×10^{-4} M. In (b), from another preparation, curare (5×10^{-4} M), applied for 15 min antagonized all the amino acid hyperpolarizations, including glycine (Gly). The concentration of all amino acids was 2×10^{-4} M. The bottom trace (Wash) was obtained 10 min after returning to a drug-free Ringer solution. Calibration in (a) also applies to (b).

mately 2.5×10^{-3} M. Atropine also completely antagonized the response to taurine (not illustrated). This selective reduction of β-alanine (and taurine) responses is identical to the action of strychnine on responses of primary afferents to amino acids (Nicoll & Barker, 1973; Barker *et al.*, 1975a). However, the relative potency of atropine was considerably less than that of strychnine, being approximately 50 times weaker.

All of the other acetylcholine antagonists tested, including dihydro-β-erythroidine, tetraethylammonium, and gallamine did not antagonize amino acid responses. The latter two agents, especially when in high concentrations (5×10^{-4} M) actually increased all of the amino acid responses by about 10-20%. The presence of acetylcholine and carbachol in the Ringer in concentrations up to 5×10^{-3} M had no effect on the amino acid responses.

Action of curare and atropine on amino acid induced hyperpolarization of motoneurones

The experiments on the primary afferent fibres indicate that curare, and to some extent nicotine, have picrotoxin-like properties while atropine has strychnine-like properties. It is of interest to know if these similarities also exist for the hyperpolarizing responses of motoneurones. Since the hyperpolarizations of frog motoneurones by neutral amino acids are more variable than the primary afferent depolarizing responses, detailed quantitative studies have not been initiated. Curare, examined in five preparations, invariably reduced the GABA and β-alanine responses in concentrations greater than 10^{-4} M but did not reduce the glutamate response (Figure 5a) or affect the d.c. potential. Glycine had a hyperpolarizing action in only two of the

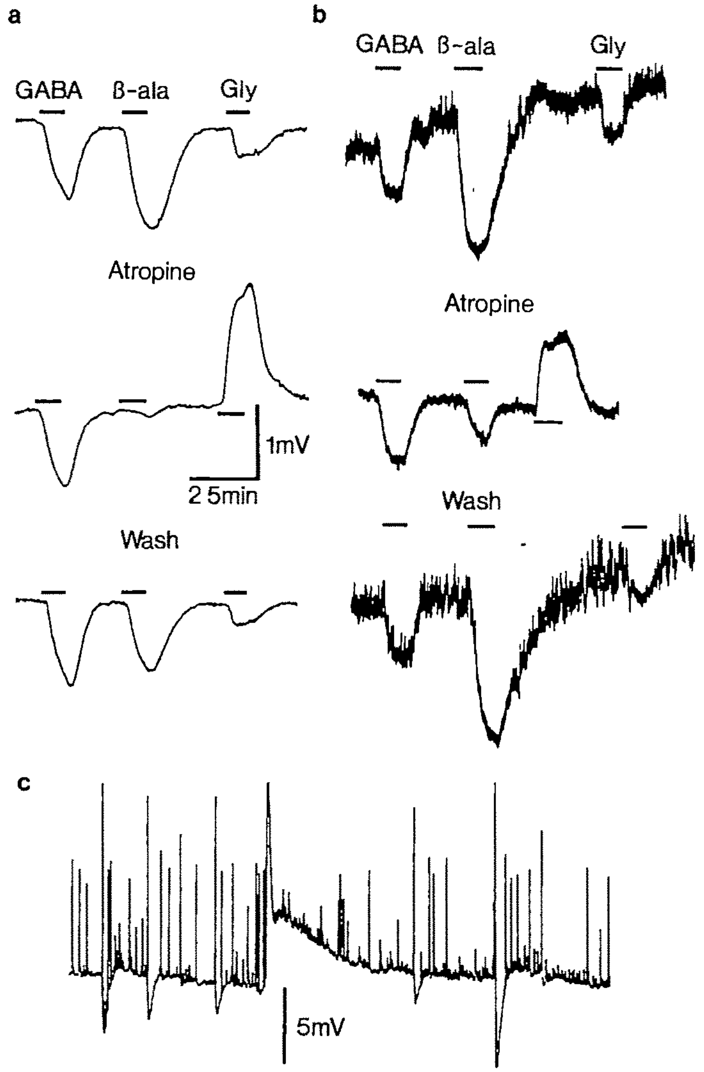

Figure 6 Atropine antagonism of amino acid hyperpolarizing responses from motoneurones. In (a) atropine (10^{-3} M), applied for 10 min, has no effect on the γ-aminobutyric acid (GABA) responses, but abolishes the β-alanine (β-ala) responses, and converts the glycine (Gly) hyperpolarization into a depolarization. The bottom trace (Wash) was obtained 20 min after washing in drug-free Ringer solution. (b) Is the same as in (a) but from another preparation in which synaptic transmission had not been blocked with magnesium ions. The concentration of atropine in this experiment was 3×10^{-3} M. The concentration of amino acids in (a) was 2×10^{-4} M and 5×10^{-4} M in (b). The calibration in (a) also applies to (b). (c) Shows the convulsant activity induced by 10^{-3} M atropine applied for 10 minutes. Time scale in (a) applies to (c).

preparations and in both cases curare antagonized the response (Figure 5b). Nicotine (4×10^{-3} M) had little effect on the hyperpolarizing amino acid responses in the two preparations tested.

The effect of atropine on amino acid hyperpolarizations was examined in four preparations. Atropine had no effect on GABA responses or d.c. potential but reduced and/or reversed the β-alanine and glycine hyperpolarizations. The action could be seen with concentrations above 10^{-4} M. The results were similar whether magnesium was (Figure 6a) or was not (Figure 6b) present to block synaptic transmission. Thus the action of atropine on the hyperpolarizing responses is similar to that obtained with strychnine (Nicoll *et al.*, 1975). In concentrations of 10^{-4}-10^{-3} M, atropine induced convulsant activity in the spinal cord when magnesium was not present which was characterized by short lasting bursts of activity (Figure 5b). In higher concentrations, however, atropine actually reduced ongoing spontaneous activity as is shown by the background in Figure 5c before and after atropine (3 x 10^{-3} M).

Discussion

A number of investigations have shown that acetylcholine and related compounds depolarize the membrane of primary afferents and motoneurones of the amphibian spinal cord (Matsuura, 1961; Phillis & Tebecis, 1967; Koketsu *et al.*, 1969). In all of these studies, however, it was difficult to exclude indirect synaptic effects. In the present study similar effects were observed with acetylcholine and carbachol. However, when synaptic transmission was blocked with magnesium ions or when action potentials were blocked with tetrodotoxin, these effects were essentially abolished even with drug concentrations of 10^{-2} M. This would imply that the membranes of motoneurones and primary afferents possess few, if any, cholinoceptors. This finding is difficult to reconcile with the proposed cholinergic recurrent excitatory pathway to motoneurones (Matsurra, 1971), although it is conceivable that recording from the spinal roots might not have detected small responses generated in the motoneurone dendrites. The fact that unmyelinated nerve terminals of the primary afferents are unresponsive to acetylcholine and carbachol suggests that the acetylcholine sensitivity observed in some unmyelinated fibres and terminals (Armett & Ritchie, 1960; Hubbard, Schmidt & Yokota, 1965; Koketsu & Nishi, 1968) does not apply to all nerve fibres.

The present results are only partially in agreement with the idea proposed at the outset, i.e., that the central excitatory effects of acetylcholine antagonists might arise from their ability to block amino acid mediated synaptic inhibition. In agreement with results obtained in the cortex (Hill *et al*, 1972) curare has the same action as picrotoxin and bicuculline on the amino acid responses evoked in primarly afferents and motoneurones. However, it is approximately 10 times weaker and somewhat less specific since it also antagonizes the glycine-induced hyperpolarization of motoneurones. Curare, like picrotoxin and bicuculline, antagonizes the dorsal root potential generated by stimulating an adjacent dorsal root (Kiraly & Phillis, 1961; Grinnell, 1966), which is thought to be mediated by GABA (Davidoff, 1972; Barker & Nicoll, 1972, 1973; Barker, Nicoll & Padjen, 1975b). Thus it seems possible that these three compounds share a common mode of action in their ability to generate convulsions, i.e., blockade of GABA-mediated synaptic potentials.

The effect of even high concentrations of nicotine ($>10^{-3}$ M) on the amino acid responses on primary afferents was weak but similar to picrotoxin, reducing the β-alanine and, to some extent, the GABA depolarizations. However, glycine depolarizations were also antagonized and hyperpolarizing components were observed with the β-alanine and glycine responses. These hyperpolarizing responses on primary afferents have not been observed with any other amino acid antagonist and raise the possibility that the responses to these amino acids in normal conditions may have a hyperpolarizing component which is masked by the more prominent depolarizing component. Indeed in a number of preparations (especially at a temperature below 18° C) the glycine response does have a hyperpolarizing component (unpublished observations; Barker & Nicoll, 1973; Barker *et al.*, 1975a). Nicotine had little effect on motoneurone hyperpolarizing responses elicited by the amino acids. Given the weak action of nicotine on the amino acid responses it would appear that the central stimulatory effects of nicotine are unrelated to amino acid-mediated synaptic inhibition. Presumably the cholinomimetic properties of nicotine play a predominant role in its central action.

Atropine behaved in a manner identical to strychnine (Nicoll & Barker, 1973; Barker *et al.*, 1975a; Nicoll *et al.*, 1975), specifically antagonizing the β-alanine responses on primary afferents and the hyperpolarizing motoneurone responses to glycine and β-alanine. This action of atropine was approximately 50 times weaker than that of strychnine. The unmasking of a depolarizing component to the glycine response on motoneurones supports the notion that there are two types of receptors for this amino acid on frog motoneurones (Nicoll *et al.*, 1975). In agreement with previous studies (Bonnet & Bremer, 1952; Phillis & Tebecis, 1967) the present results indicate that atropine can induce convulsant activity in the spinal cord, but in concentrations greater than 10^{-3} M, it tends to suppress ongoing activity. It is quite possible that in high concentrations the local anaesthetic action of atropine (Curtis & Phillis, 1961) tends to counteract its convulsant properties. The observation that atropine does not block strychnine-sensitive post-synaptic inhibition in the cat spinal cord (Curtis & Duggan, 1967) presumably arises from the much lower concentration used.

The fact that dihydro-β-erythroidine does not antagonize amino acid responses and yet is a competitive antagonist of acetylcholine suggests that the amino acid antagonist properties of curare, and possibly atropine, may be unrelated to the properties they share in relation to acetylcholine receptors.

The failure of tetraethylammonium and gallamine to antagonize amino acid responses indicates that their well documented excitatory effects arise from some property other than that of blockade of amino acid-mediated synaptic inhibition. Both of the compounds are known to block the delayed increase in K^+-conductance during an action potential, which can prolong the action potential, leading to repetitive neuronal firing (Washizu, 1959; Grinnell, 1970) and enhanced transmitter release (Koketsu, 1958; Payton & Shand, 1966). This property could account for some of the observed central excitatory effects of these two compounds. Clearly the present results do not exclude the possibility that some of the excitatory actions of acetylcholine antagonists might, in fact, arise from blockade of cholinergic pathways although acetylcholine is generally considered to be an excitatory transmitter. Acetylcholine responses and the dorsal root potential generated by ventral root stimulation (VR-DRP), which is thought to contain a cholinergic synapse, appear to have a sensitivity to acetylcholine antagonists (Kiraly & Phillis, 1961, Grinnell, 1966; Koketsu *et al.*, 1969) similar to that of the amino acid responses in the present study.

The ability of curare, nicotine, and atropine to antagonize amino acid responses on primary afferents could contribute to the antagonism these compounds exert on the VR-DRP, which occurs at similar concentrations, since amino acids are thought to be involved in the final step in this pathway (Davidoff, 1972; Nicoll & Barker, 1973; Barker *et al.*, 1975b). However, the inability of tetraethylammonium gallamine, and dihydro-β-erythroidine to antagonize amino acid responses and yet block the VR-DRP (Kiraly & Phillis, 1961; Grinnell, 1966; Koketsu *et al.*, 1969) clearly indicates that there is a cholinergic step in this pathway.

The generous research support by Dr Henry C. and Bertha H. Buswell Fund is greatly appreciated. This work was supported by a grant from the National Institutes of Neurological Diseases and Stroke, RO1NB0822101, 2, 3, 4.

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(Received November 7, 1974.
Revised July 22, 1975.)

Br. J. Pharmac. (1975), 55, 459-473

CENTRAL EFFECTS OF CLONIDINE 2-(2,6-DICHLOROPHENYLAMINO)-2-IMIDAZOLINE HYDROCHLORIDE IN FOWLS

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1 The effects of clonidine infused into the IIIrd cerebral ventricle, the hypothalamus or intravenously were studied on behaviour, electrocortical activity, body, comb and leg temperatures, respiration and carbon dioxide elimination in adult and young fowls (*Gallus domesticus*).

2 Behavioural and electrocortical slow wave sleep were induced by clonidine infused into IIIrd cerebral ventricle, the hypothalamus or intravenously. Surprisingly, sleep elicited by intravenous clonidine was much longer-lasting than that induced by an identical dose given intraventricularly.

3 Body temperature was lowered by clonidine given intraventricularly or infused into the hypothalamus. Depending on initial comb temperature and ambient temperature, comb temperature was elevated, unaffected or lowered as body temperature fell; temperature of the unfeathered legs also rose as body temperature declined after clonidine.

4 Following clonidine, but before any considerable decline of body temperature, tachypnoea and wing abduction developed; during recovery of body temperature, the wings were lowered and applied closely to the trunk and the feathers partly erected.

5 CO_2 elimination fell more swiftly than body temperature following intrahypothalamic clonidine in young chicks; initial recovery developed sooner than that of body temperature, but eventual recovery was delayed compared to that for body temperature. The effects of clonidine were much more marked in young chicks studied at an ambient temperature below thermoneutrality as compared to thermoneutrality.

6 The soporific effects of clonidine were attenuated by intraventricular phentolamine; its hypothermic effects were prevented by phenoxybenzamine and prevented or attenuated by phentolamine. Intraventricular atropine, haloperidol, methysergide and propranolol were ineffective.

7 Larger doses of intraventricular phentolamine elicited shivering, tachypnoea and wing abduction; body temperature was elevated, to the extent even of lethal hyperthermia. Intraventricular atropine also elevated body temperature.

8 Clonidine infused intravenously, intraventricularly or into the hypothalamus, replaced the behavioural and electrocortical arousal evoked with dexamphetamine, by sleep associated with slow wave electrocortical activity.

Introduction

Clonidine has sympathomimetic properties ascribed to an action on peripheral and central α-adrenoceptors (Nayler, Price, Swann, McInnes, Race & Lowe, 1968; Holman, Shillito & Vogt, 1971). It readily crosses the blood-brain barrier and given intravenously, induced sleep in adult rats and cats (Holman *et al.*, 1971). This was an important finding because of the controversy as to the significance of sleep produced by noradrenaline given into the brain; noradrenaline also acts on peripheral α-adrenoceptors but does not cross the blood-brain barrier, except in young chickens. Intravenous clonidine induced sleep in young chicks (Zaimis, 1970) indistinguishable from normal sleep and that elicited by intravenous noradrenaline or adrenaline (Holman *et al.*, 1971);

the hypnotic potency of clonidine was 25 or 30 times that of noradrenaline. Effects of intraventricular clonidine on body temperature of sheep and goats, exposed to different ambient temperatures, were similar to those resulting with larger intraventricular doses of noradrenaline (Maskrey, Vogt & Bligh, 1970).

In the present experiments, central effects of clonidine were studied by infusing it directly into the brain (IIIrd ventricle, hypothalamus) of young and adult fowls, and compared, where necessary, with those of noradrenaline infused into the same brain areas. Despite the similar spectrum of effects elicited by the two drugs, their profiles differed, activation of heat-loss mechanisms, for example, being much more evident with clonidine. A preliminary account of the work has been published (Marley & Nisticò, 1974).

Methods

Adult or young Rhode Island Red pullets were used. Adults (1.75-3.0 kg) were kept at room temperature (20°-25°C). Young chicks (16-18 days, approx. 85 g) were maintained at a cage temperature of 25°-31°C.

Operative and testing procedures

Recovery anaesthesia with halothane, the stereotactic implantation of infusion cannulae into the IIIrd cerebral ventricle and hypothalamus, the brain coordinates for these cannulae, implantation of a thermistor beneath the feathered skin of the interscapular region, methods for preparing and implanting cortical recording electrodes together with post-operative care, were as previously described (Marley & Nisticò, 1972). So also were methods for measuring body temperature (except in the case of leg temperature which was recorded from a thermistor taped to the unfeathered tarsometatarsal region of a hind limb), and for respiratory rate. Fowls were tested in a chamber, the ambient temperature of which was maintained at some point between 17° and 26°C (the lower and upper thermoneutral limits for adult fowls given by Barott & Pringle, 1946 and King & Farmer, 1961). For infusions via the implanted cannulae, those into the hypothalamus were in a volume of 1.0 μl or less, and those into the IIIrd cerebral ventricle of 10 μl or less. Histological preparation of the brains was also as previously described. Adult fowls were not tested until at least a week following recovery from operative procedures, and thereafter at intervals of at least a week. They were placed in the test-chamber between 9 h 00 min and 10 h 00 min and one hour's control body temperature recorded before drugs were administered.

The technique for measuring CO_2 elimination is that described by Marley & Stephenson, 1975. The chick was placed in a 1 litre chamber, maintained at 31° or 16°C, through which CO_2-free air, maintained respectively at 31° or 16°C, was passed at 1 litre/minute. Percentage CO_2 in the expired air, measured with an infra-red CO_2 analyser (Hartmann & Braun), was recorded continuously. These chickens were tested at least 24 h after operative procedures when recovery was complete; only chickens which had fed since the operation, as indicated by presence of food in the crop, were used. Ambient temperature of 31°C is within, and 16°C below the thermoneutral range for chicks of this age (Freeman, 1963; Allen & Marley, 1967); relative humidity was maintained at approximately 60%.

Drugs

The following drugs were used: the hydrochlorides of clonidine, haloperidol (Serenace, Searle), phentolamine (Regitine, Ciba) and (±)-propanolol; atropine sulphate, mebanazine oxalate and methysergide bimaleate. (−)-Noradrenaline base was dissolved immediately before use in equimolar HCl. The pH of the clonidine solutions was 6.4 to 6.5.

Results

Infusion of clonidine into the IIIrd ventricle

Behaviour and electrocortical activity Clonidine (0.02, 0.04, 0.05, 0.1, 0.2, 0.25, 0.4 μmol) induced behavioural and electrocortical sleep within 5 to 10 min of infusion and lasting approximately 20-60 min, depending on dose. This was followed by a period in which drowsy and alert states alternated (Figure 1). During sleep, fowls stood or squatted. The change from the control small amplitude, fast frequency, alert electrocorticogram (Figure 1a) to that of the larger amplitude, slow frequency sleep pattern (Figure 1b) after clonidine was associated with a two- to threefold increase of electrocortical integrals (Figure 1c). During clonidine-induced sleep, sensory stimuli induced behavioural and phasic electrocortical arousal.

Respiratory rate and wing abduction Clonidine evoked tachypnoea and abduction of the wings about 45° from the trunk, both heat-loss mechanisms. As indicated in Figure 1d, these developed within a few minutes of intraventricular

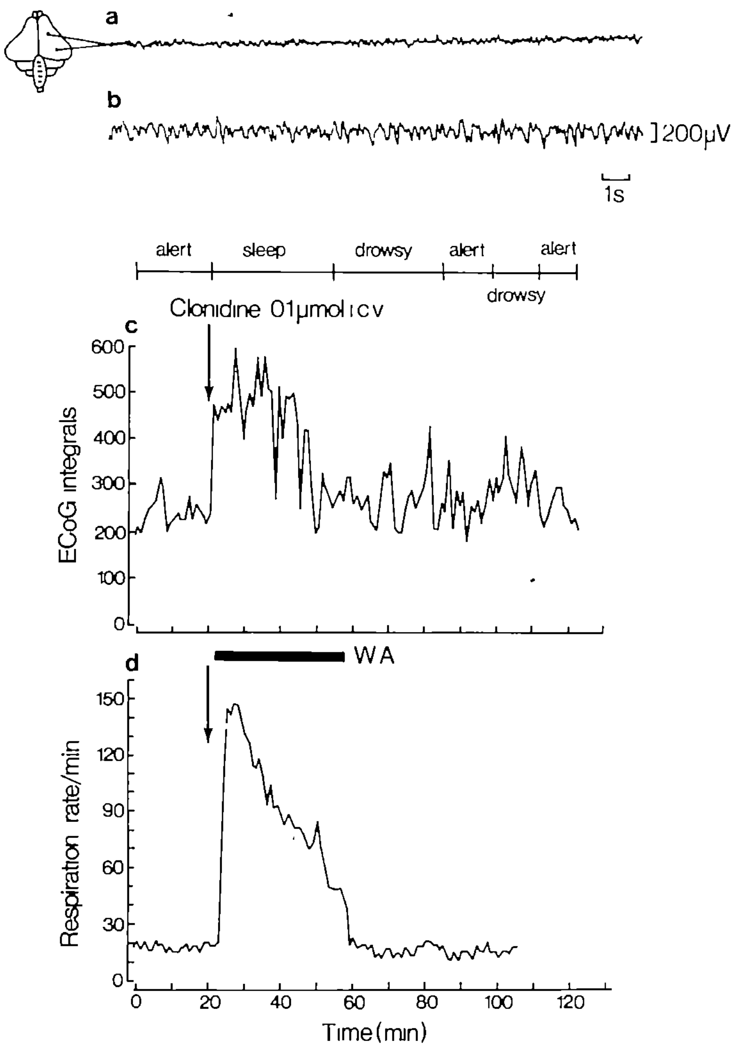

Figure 1 Records of electrocortical activity (a, b), histogram of integrated electrocortical activity (c) and graph of respiratory rate (d) in an unanaesthetized adult fowl. Solid bar indicates duration of wing abduction (W.A.). (a) Control alert electrocortical activity. (b) Slow frequency (4-7 Hz), large amplitude electrocortical potentials 15 min after clonidine, 0.1 µmol, infused into the IIIrd cerebral ventricle (i.c.v.). (c) Electrocortical integrals showing marked increase (up to a peak of 600/min) following intraventricular infusion of clonidine and associated with behavioural sleep. (d) Graph of respiratory rate illustrating increase from control values of 15-20/min up to a peak of 147/min following clonidine. Period of increased respiratory rate corresponds approximately to the duration of wing abduction.

clonidine, 0.1 µmol, a time when body temperature had fallen only 0.2-0.3°C, and persisted 40 min; tachypnoea in excess of 60-90/min was accompanied by gular flutter. These rapid respiratory rates did not disturb the induced sleep. Once wing abduction abated, the wings were lowered and applied closely to the trunk and lower limbs, the body feathers were erected and the tail flexed, procedures reducing heat loss.

Body, comb and leg temperatures Clonidine, 0.02, 0.05, 0.2 µmol, elicited dose-dependent falls in body temperature of 0.8°, 1.4°, 1.4° and 2.0°C respectively (Figure 2); duration of effect was less

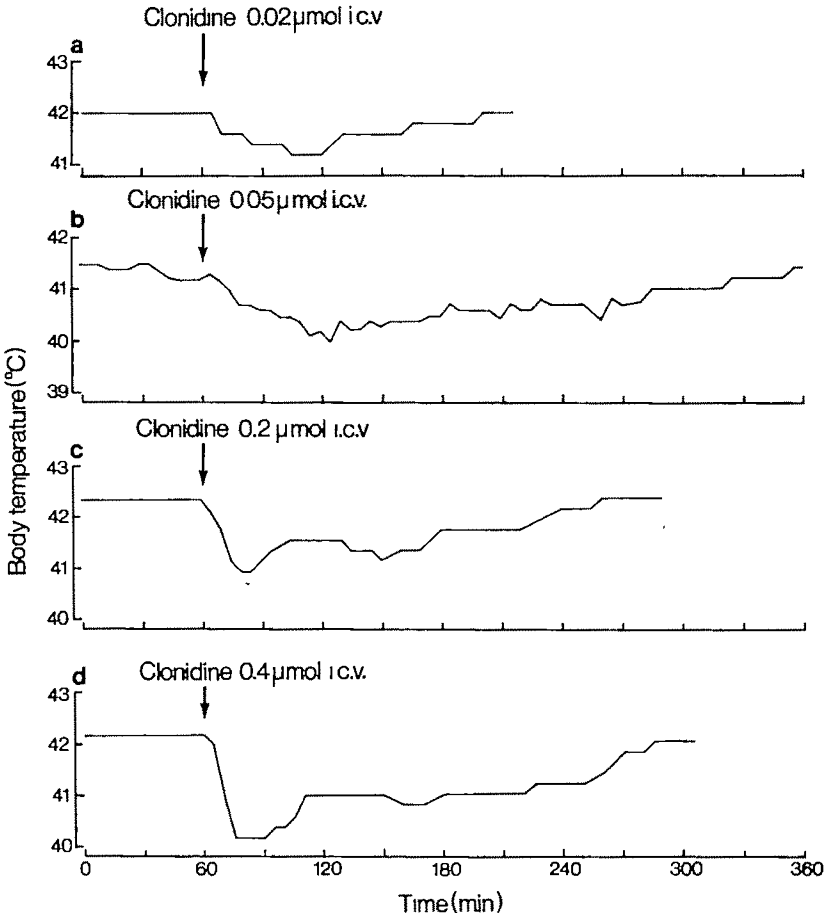

Figure 2 Records of body temperature from same unanaesthetized fowl, tested at weekly intervals, to illustrate responses to four different intraventricular (i.c.v.) doses of clonidine (0.02 µmol, 0.05 µmol, 0.2 µmol, 0.4 µmol). Ambient temperature in four experiments was 20° C.

convincingly correlated to the size of the dose. With an ambient temperature in the lower half of the thermoneutral range (17°-22° C), comb temperature was low (27°-30° C) and clonidine induced reciprocal changes in comb and body temperatures. Thus comb temperature rapidly increased by up to 10° C after clonidine 0.1 µmol, attaining its zenith before body temperature reached its nadir, the increase being maintained until body temperature recovered (Figure 3a, b). With an ambient temperature at the upper limit of thermoneutrality (25°-26° C), comb temperature was initially high (35°-37° C) and not even a large dose of clonidine (0.4 µmol), elevated comb temperature, despite lowering body temperature 1.6° C; indeed comb temperature was lowered 5°-6° C (2 of 4 fowls) or was unaltered (2 of 4 fowls).

Skin temperature of the unfeathered region of the legs Following intraventricular clonidine, 0.1 µl (2 fowls), leg temperature rose from 27°-29° C to 34°-36° C in the erect fowl, body temperature declining up to 1.4° C. However, since a similar elevation of leg temperature occurred when the fowl squatted and since sleep induced by clonidine was accompanied at some stage by squatting, this measure had limited usefulness.

Infusion of clonidine into the hypothalamus

Behaviour and electrocortical activity Eight fowls were tested and sleep consistently induced with clonidine 0.02, 0.04, 0.05 and 0.1 µmol. This developed after 5 to 10 min and lasted 30-100 min, although fowls were easily roused by sensory stimuli. Sleep was more readily induced by

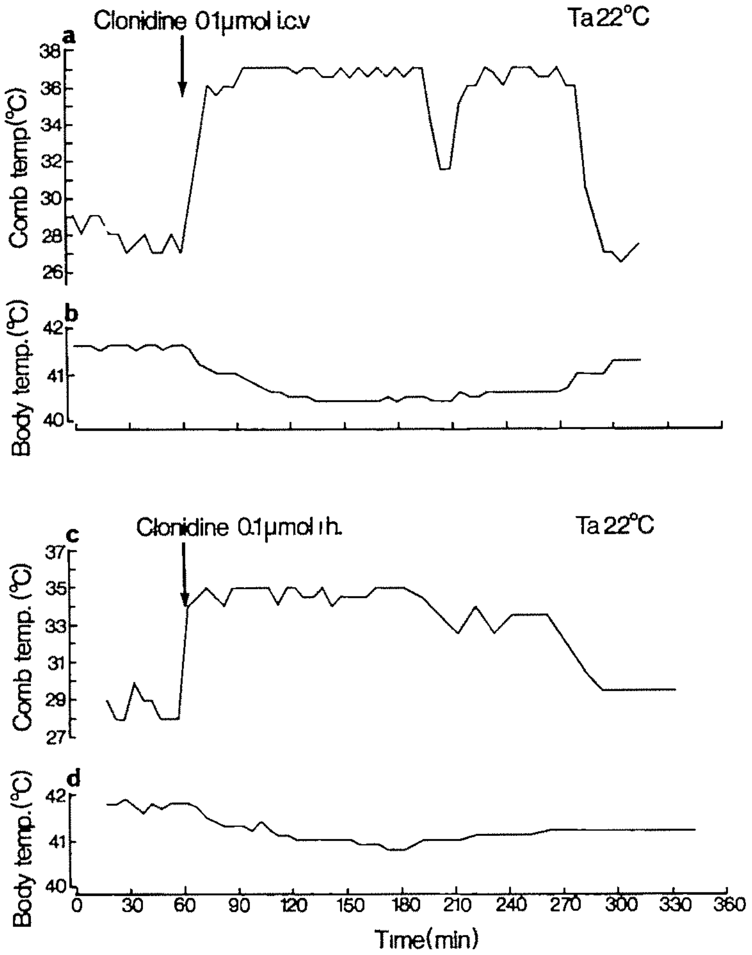

Figure 3 Records from above downwards of comb and body temperatures of two unanaesthetized fowls (a, b; and c, d respectively) to illustrate effects of clonidine infused into the IIIrd cerebral ventricle (a, b) and hypothalamus (c, d), ambient temperature (Ta) being 22° C in both experiments. (a and b) clonidine, 0.1 µmol, infused intraventricularly (i.c.v.) and in (c and d) clonidine, 0.1 µmol, infused into the hypothalamus (i.h.), promptly elevated comb temperature but lowered body temperature more slowly. Comb and body temperatures recovered *pari passu.*

infusion into the posterior rather than the anterior hypothalamus. Postural and electrocortical changes were similar to those after intraventricular clonidine.

Intrahypothalamic clonidine, unlike dopamine (Marley & Nisticò, 1972) did not elicit head movements. The soporific and hypothermic effects were not prolonged by mebanazine (100 µmol/kg i.m. 18 h, 6 h and 30 min previously).

Respiratory rate and wing abduction Within 5 min of infusion into the posterior hypothalamus of two fowls, clonidine, 0.1 µmol, increased respiratory rate, maxima of 108 breaths/min and 124 breaths/min being attained, with recovery after 50 min and 25 min respectively; gular flutter developed when respiratory rate exceeded 60/minute. In two fowls, in which the cannula-tip was more anterior, respiratory rate increased after clonidine, 0.1 µmol, but maxima of 50/min and 32/min only were achieved. Wing droop developed in the five fowls tested, preceded by wing abduction in three, the latter commencing 5-10 min after infusion and lasting 15, 45 and 70 min respectively.

Body and comb temperatures Individual maximal falls in body temperature obtained after infusing

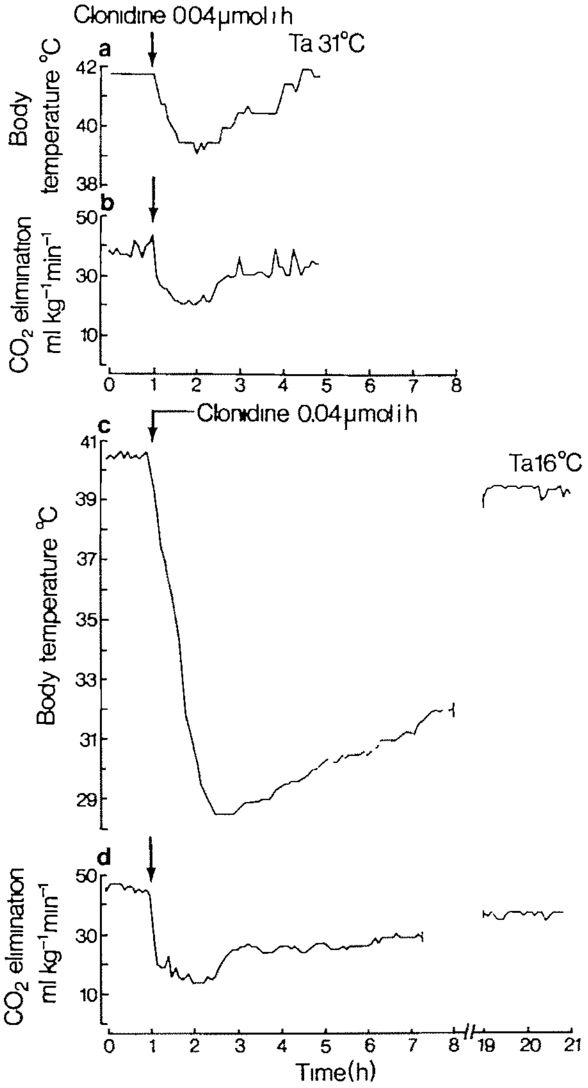

Figure 4 Effects of clonidine infused into the hypothalamus (i.h.) on body temperature and CO_2 elimination in two chicks (a, b and c, d respectively) at ambient temperatures (Ta) of thermoneutrality (31° C) and below thermoneutrality (16° C). Clonidine, 0.04 µmol, immediately lowered CO_2 elimination followed by a decline in body temperature. Onset of recovery of CO_2 elimination preceded that of body temperature although complete recovery was slower. Note effects of clonidine were much more pronounced on body temperature and CO_2 elimination at an ambient temperature of 16° C than at 31° C.

clonidine into the hypothalamus of adult fowls were as follows: clonidine 0.02 µmol, 0.6° and 1.4° C; 0.04 µmol, 1.2°, 1.5° and 1.6° C; 0.1 µmol, 0.8°, 1.0°, 1.3° and 1.7° C. Thus maximal fall of body temperature did not correlate with dose, nor did it necessarily exceed that obtained with the same dose given intraventricularly. However, whereas body temperature usually recovered within 2.5 to 4 h of intraventricular clonidine, recovery was delayed as much as 7 h with intrahypothalamic infusions, and in 3 of 4 fowls given clonidine, 0.1 µmol, body temperature was restored after 7 h only by raising ambient temperature.

With an ambient temperature in the lower half of the thermoneutral range (17°-22°C), comb

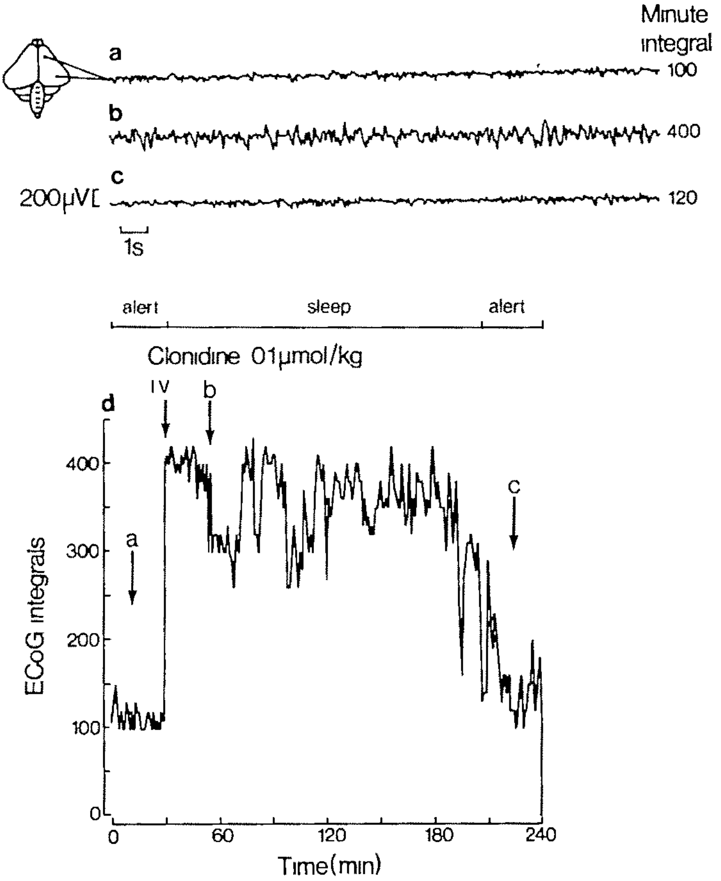

Figure 5 Records of electrocortical activity (a-c) and histogram of integrated electrocortical activity (d) in an unanaesthetized adult fowl. (a) Control alert electrocortical activity. (b) Slow frequency (4-7 Hz), large amplitude electrocortical sleep potentials 24 min after intravenous clonidine, 0.1 μmol/kg. (c) Alert electrocortical activity on recovery from sleep induced by clonidine. (d) Electrocortical integrals showing increase during sleep induced by intravenous clonidine, with subsequent reduction once sleep abated.

temperature was initially low (27°-29° C) and clonidine induced reciprocal changes in body and comb temperatures. Thus following clonidine, 0.1 μmol, comb temperature rose rapidly to 35° C (Figure 3c) as body temperature fell slowly (Figure 3d). This reciprocal relation continued during recovery. In contrast, with an ambient temperature at the upper limit of thermoneutrality (25°-26° C), comb temperature was initially high (36°-37° C) and clonidine, 0.1 μmol, despite lowering body temperature up to 1.7° C was initially without effect on comb temperature, although it subsequently fell 3°-4° C when body temperature was at its nadir.

CO_2 elimination and body temperature These experiments were performed on 16 and 18 day chicks at, and below, thermoneutral ambient temperatures. At a thermoneutral ambient temperature of 31° C (as shown in Figure 4a), clonidine, 0.04 μmol, infused into the hypothalamus lowered body temperature 2.6° C, 60 min later, with recovery at 200 min; sleep was induced for 40 minutes. CO_2 elimination declined from a mean of 39 ml kg^{-1}min^{-1} in the 60 min before clonidine, to 21 ml kg^{-1}min^{-1} 40 min after infusion, and recovered to a mean of 34.4 ml kg^{-1}min^{-1} 2 to 3 h subsequently (Figure 4b).

At an ambient temperature below thermoneutrality (16° C), the effects of clonidine, 0.04 μmol, infused into the hypothalamus were much intensified and prolonged. Thus, maximum fall in body temperature was 11.9° C, reached

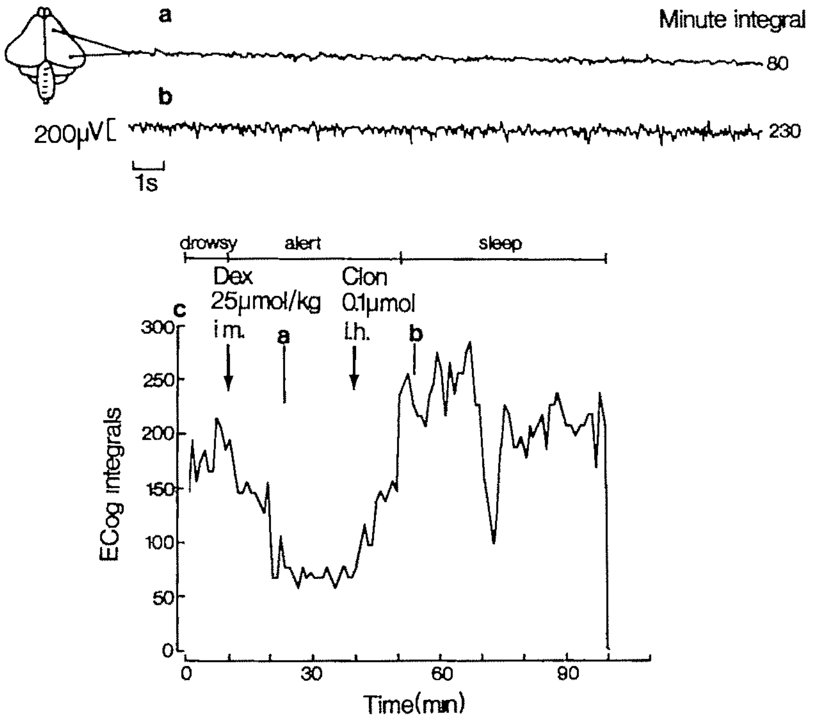

Figure 6 Records of electrocortical activity (a, b) and histogram of integrated electrocortical activity (c) in an unanaesthetized adult fowl. (a) Alert electrocortical activity induced by dexamphetamine (Dex 25 µmol/kg i.m.) injected 13 min previously. (b) Slow frequency (7-12 Hz), large amplitude electrocortical potentials, 14 min after clonidine (Clon), 0.1 µmol, infused into the hypothalamus (i.h.). (c) Electrocortical integrals showing decrease following dexamphetamine, with subsequent increase accompanying behavioural and electrocortical sleep.

90 min after clonidine and recovery was incomplete even 21 h later (Figure 4c). Mean CO_2 elimination was elevated compared to that at 31°C, averaging 45.5 ml $kg^{-1}min^{-1}$ for the control period. CO_2 elimination decreased significantly within 5 min of clonidine infusion, at a time when body temperature had fallen merely 0.3°C, and its rate of decline continued more swiftly than that for body temperature, a nadir of 14 ml $kg^{-1}min^{-1}$ being reached 60 min after infusion (Figure 4d). At 100 min after clonidine, when body temperature was at its lowest point, CO_2 elimination had begun recovery (Figure 4d), although this was still incomplete 19 h later.

Intravenous injection of clonidine

Intravenous clonidine also produced behavioural and electrocortical sleep, which were comparatively enduring. Thus intravenous clonidine, 0.05 µmol/kg (total 0.1 µmol) induced sleep lasting 90 min, whereas its duration was 40 min after clonidine, 0.1 µmol, given intraventricularly. The change from the control alert to the sleep electrocorticogram after clonidine, 0.1 µmol/kg, intravenously is shown in Figure 5a, b; electrocortical integrals increased from 100-150/min to 260-435/min (Figure 5d). Respiratory rate rose from 20/min to 122/min within 2 min of clonidine, 0.05 or 0.1 µmol/kg, intravenously and remained elevated for 6 min, subsiding to normal 16 min later.

Reversal of dexamphetamine arousal by clonidine

Clonidine given intravenously, intraventricularly, or into the hypothalamus, replaced dexamphetamine-induced behavioural and electrocortical arousal with sleep. Thus, as shown in Figure 6, once arousal had been established for 30 min by dexamphetamine 25 µmol/kg intramuscularly, and which would have customarily lasted 2 h, clonidine, 0.1 µmol, was infused into the hypothalamus; behavioural and electrocortical (Figure

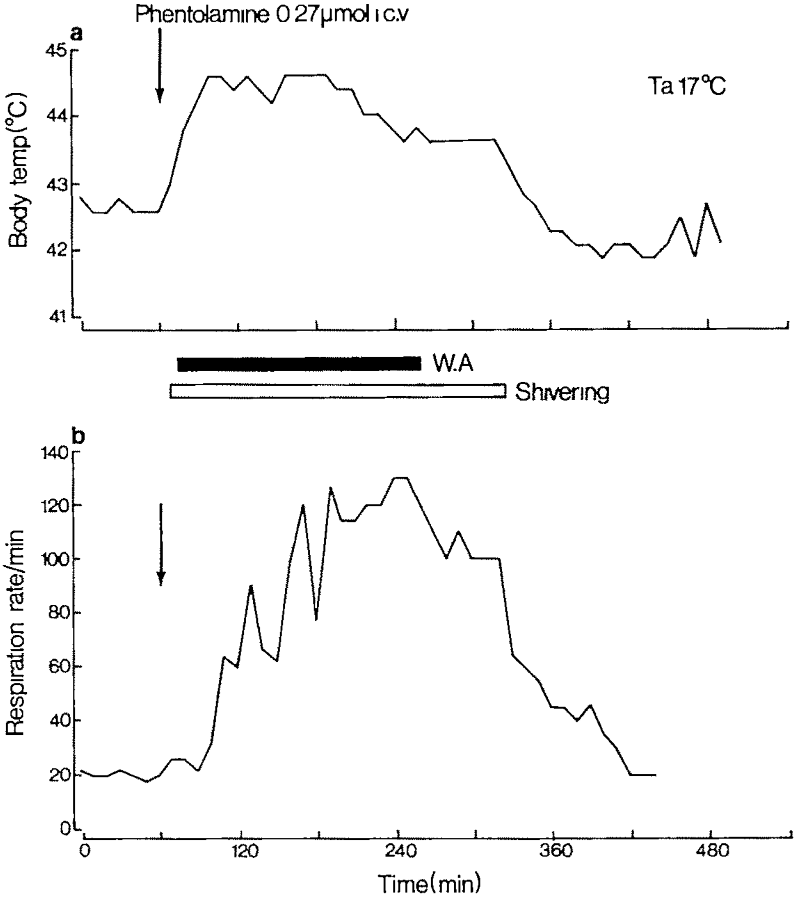

Figure 7 Records from above downwards of body temperature (a), and respiratory rate (b) in an unanaesthetized adult fowl in which phentolamine was infused intraventricularly (i.c.v.). Solid bar, and open bar, duration of wing abduction (W.A.) and shivering respectively. (a) Elevation of body temperature and (b) of respiratory rate following intraventricular infusion of phentolamine, 0.27 μmol, associated with shivering and wing abduction. Once shivering and wing abduction abated, body temperature and respiratory rate returned to pre-infusion values.

6b) sleep, accompanied by increase in electrocortical integrals (Figure 6c) developed after 11 minutes.

Antagonism of clonidine

Delbarre & Schmitt (1971) and Holman *et al.* (1971) noted that the soporific effects of clonidine were attenuated by intramuscular phentolamine. The interaction between clonidine and centrally applied phentolamine was therefore studied.

Intraventricular phentolamine was tested over a dose range 0.05 to 0.27 μmol. As indicated in Figure 7 intraventricular phentolamine, 0.27 μmol, elevated body temperature 1.9° C, palpable and visible shivering of the trunk and lower limbs commencing after about 10 min and wing abduction at 15 min; respiratory rate increased more slowly to a peak of 130/min (Figure 7b). Respiratory rate and body temperature reverted to normal, once shivering and wing abduction abated. Intraventricular phentolamine, 0.135 μmol, had similar effects, body temperature increasing 2.3° C to reach 44.5° C, but the fowl then became comatose and died. Smaller intraventricular doses of phentolamine were therefore administered before infusion of clonidine into the hypothalamus. To assess whether antagonism to clonidine was pharmacological, or merely the resultant of

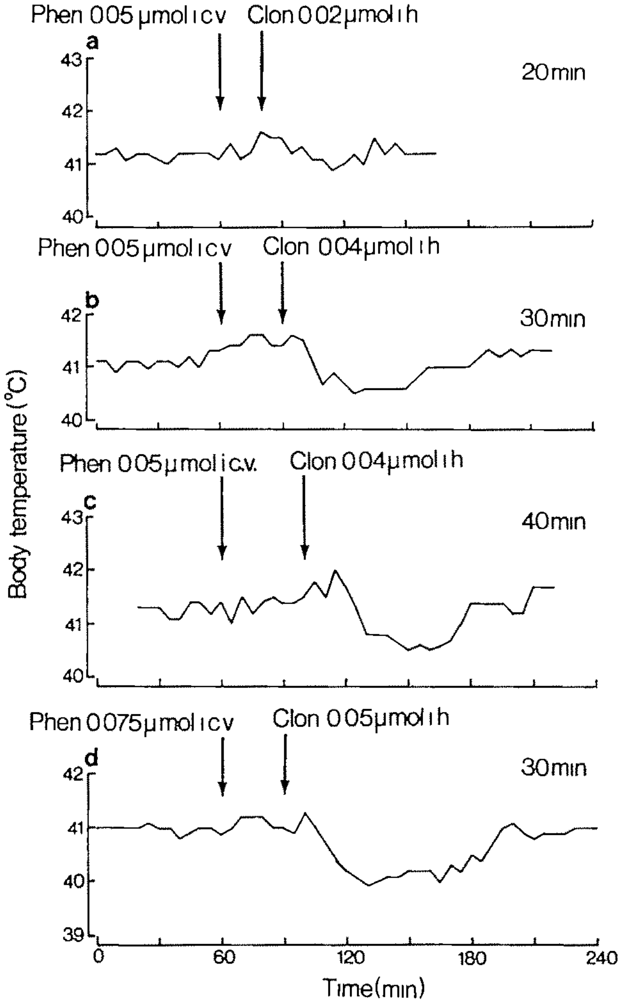

Figure 8 Records of body temperature from four adult unanaesthetized fowls in which phentolamine (Phen), 0.05 µmol or 0.75 µmol, was infused intraventricularly (i.c.v.), followed 20, 30 and 40 min subsequently by clonidine (Clon) infused into the hypothalamus (i.h.) (respectively, one dose for each fowl, 0.02 µmol, 0.04 µmol, 0.04 µmol and 0.05 µmol). There was antagonism of the hypothermic effects of clonidine 0.02 µmol. Clonidine lowered body temperature in the other three fowls, but its effects were markedly attenuated. Ambient temperature in range 21° -22° C for the four experiments.

the hyperthermic effects of phentolamine offset against the hypothermic actions of clonidine, the latter was injected in different experiments at different times after phentolamine (Figure 8). The results, except perhaps as indicated in Figure 8c in which body temperature increased 0.6°C after phentolamine, but before the hypothermic effects of clonidine developed, would argue for pharmacological antagonism. Certainly, duration of the hypothermic effects elicited with clonidine, 0.04 and 0.05 µmol (Figure 8b, d), which would otherwise have persisted for up to 7 h, were much attenuated. In confirmation, the control response to clonidine, 0.04 µmol, in the same fowl from which Figure 8c was taken, amounted to a fall in body temperature of 1.6°C with incomplete recovery 5 h after the infusion (Figure 9a), whereas following phentolamine, the same dose lowered body temperature 0.7°C with recovery 2 h after clonidine (Figures 8c, 9b). Intraventricular phenoxybenzamine, 0.25 µmol, itself without effect on body temperature, prevented

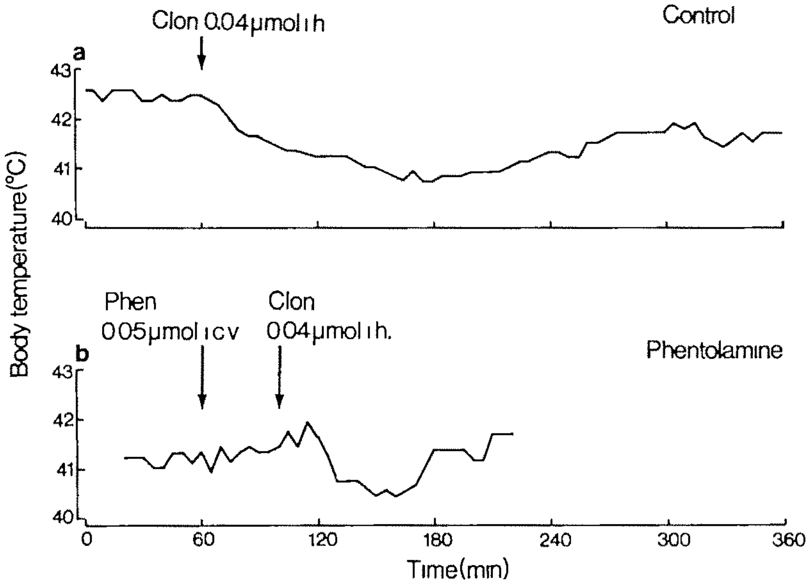

Figure 9 Records of body temperature from an adult unanaesthetized fowl. (a) Control response to clonidine (Clon), 0.04 µmol, infused into the hypothalamus (i.h.). (b) Hypothermic effect of this dose of clonidine was much reduced in duration and intensity by the intraventricular (i.c.v.) injection of phentolamine (Phen), 0.05 µmol, 40 min previously. (Experiment (b) preceded by 7 days experiment (a).)

the hypothermic effects of intrahypothalamic clonidine, 0.5 µmol. After phentolamine, sleep was not obtained with clonidine, 0.02 µmol, and was reduced in duration with the 0.04 and 0.05 µmol doses (experiments Figure 8b, d).

In other tests, antagonists were given intraventricularly 25 min before intraventricular clonidine. Atropine 0.4 µmol, haloperidol 0.4 µmol, methysergide 0.1 µmol, and propranolol 0.25 µmol, did not antagonize the soporific, hypothermic, respiratory and postural effects evoked by intraventricular clonidine 0.05, 0.08 and 0.2 µmol.

Noradrenaline

In earlier experiments (Marley & Nisticò, 1972), noradrenaline and other catecholamines, infused intraventricularly or into the hypothalamus of chickens produced sleep, diminished body temperature and evoked postural changes, amongst which lowering of the wings was conspicuous; increased respiratory rate was noted only after intraventricular isoprenaline or dopamine (see Discussion section). Noradrenaline was therefore retested, to ascertain whether tachypnoea and wing abduction were elicited and how comb and body temperatures altered.

Respiratory rate and wing abduction Following intraventricular noradrenaline, 0.5 µmol (2 fowls), respiratory rate increased from control values of 16-20/min to maxima of 56/min and 48/min respectively, the increases lasting 25 to 40 min respectively. Following intrahypothalamic noradrenaline, 0.1 µmol (1 fowl), respiratory rate increased to 124/min and was elevated for 20 minutes. Wing abduction developed in the three fowls.

Body and comb temperature With an initially low comb temperature (30° C) and an ambient temperature in the middle of the thermoneutral range (22° C), intraventricular noradrenaline, 1.0 µmol, elevated comb temperature 5.0° C (Figure 10a), with return to pre-infusion values 70 min later, body temperature falling 1.6° C (Figure 10). In contrast, when comb temperature was high (36°-37° C) and ambient temperature still 22° C, intraventricular noradrenaline, 0.5 µmol, lowered comb temperature 5° C and body temperature 1.4° C (Figure 10c, d); recovery of these temperatures occurred *pari passu*. With ambient temperature at the upper thermoneutral limit (25°-26° C) and comb temperature high (35°-36° C), noradrenaline, 0.5 µmol, infused intraventricularly or 0.1 µmol, infused into the hypo-

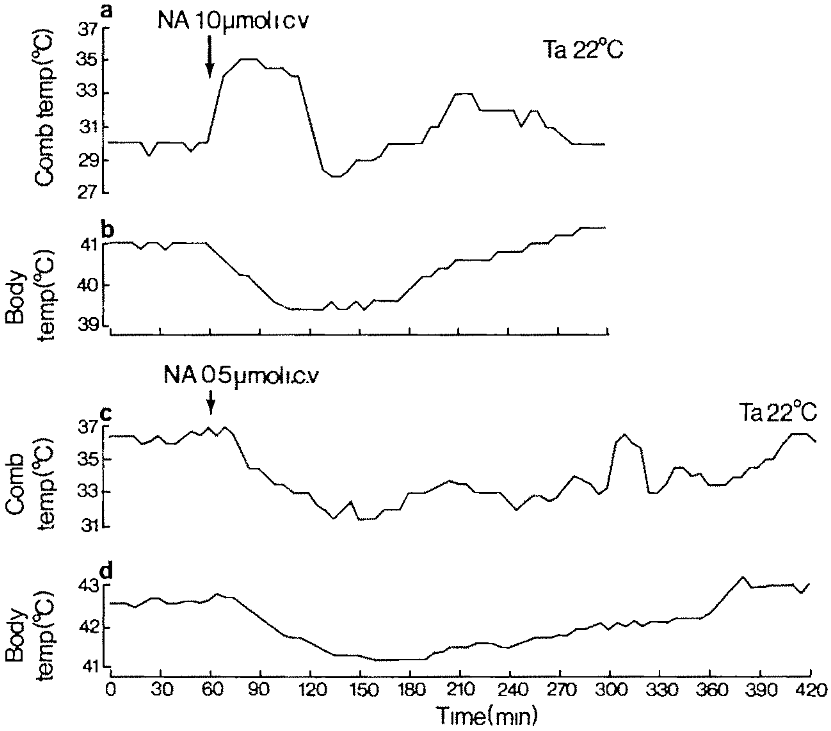

Figure 10 Records from above downwards of comb and body temperatures of two unanaesthetized fowls (a, b and c, d respectively) to illustrate effects of noradrenaline (NA) infused into the IIIrd cerebral ventricle, ambient temperature (T_a) being 22° C in both experiments (a and b) Comb temperature initially low. Noradrenaline, 1.0 μmol, infused intraventricularly (i.c.v.) initially elevated comb temperature but lowered body temperature. (c and d) Comb temperature initially high. Noradrenaline, 0.5 μmol, infused intraventricularly (i.c v.) lowered both comb and body temperatures.

thalamus, although depressing body temperature up to 1.6° C, scarcely affected comb temperature, lowering it 2° C at most.

Discussion

Clonidine infused into the IIIrd cerebral ventricle or hypothalamus of fowls induced behavioural and slow wave electrocortical sleep and a fall in body temperature, as did catecholamines. Smaller doses of clonidine were required than of noradrenaline, although on an equimolar basis, sleep was briefer with clonidine whereas the hypothermic effects at thermoneutrality were longer lasting. Remarkably, in adult fowls, intravenous clonidine induced much more prolonged behavioural and slow wave electrocortical sleep than was obtained with an identical dose given intraventricularly; since clonidine crosses the blood-brain barrier, access to brain areas involved in sleep was clearly superior from the blood than from the cerebrospinal fluid. Sleep following clonidine appeared to be due to an action on central α-adrenoceptors, since it was attenuated by phentolamine but not by intraventricular doses of antagonists at β-adrenoceptors or to 5-hydroxytryptamine, dopamine or acetylcholine. Holman *et al.* (1971) concluded that clonidine evoked sleep in young chicks by an action on central α-adrenoceptors. There is evidence that central α-adrenoceptors, mediating clonidine-induced sleep, differ from those in the periphery (Delbarre & Schmitt, 1973).

The findings with clonidine reinforce the notion that noradrenaline, the neurotransmitter acting on central α-adrenoceptors, is important for sleep associated with slow wave electrocortical activity, despite such emphatic claim that 5-hydroxytryptamine is the amine involved. Indeed, sleep with slow wave electrocortical activity was induced in fowls both by centrally applied noradrenaline (Grunden & Marley, 1970; Marley & Stephenson, 1970; Marley & Nisticò, 1972) or 5-hydroxytryptamine (Marley & Nisticò, 1975; Marley & Whelan, 1975a), providing grounds for assuming involvement of noradrenergic as well as 5-hydroxytryptaminergic systems. Since clonidine elicited sleep in rats previously rendered awake by *p*-chlorophenylalanine, Holman *et al.* (1971) concluded it was not mediated through the ascending 5-hydroxytryptaminergic system. 'Wakefulness' induced by *p*-chlorophenylalanine is one of the strongest pieces of evidence for implicating 5-hydroxytryptamine in electrocortical slow wave sleep; however, any assumption that this can be equated with normal wakefulness has been vigorously criticized (Rechtschaffen, Lovell, Freedman, Whitehead & Aldrich, 1973), and to complicate interpretation, *p*-chlorophenylalanine has pharmacological actions other than tryptophan hydroxylase inhibition (Marley & Whelan, 1975b).

Despite similarities in sleep provoked by clonidine and noradrenaline, there were differences in their other effects, possibly because of clonidine's greater potency. Thus, activation of heat-loss mechanisms was much more evident with clonidine. For example, and not previously reported in the fowl or other species, clonidine evoked marked tachypnoea (respiratory heat loss) at thermoneutral ambient temperatures; wing abduction and less constantly, increase in comb and leg temperatures developed, all promoting radiant heat loss. These changes were evident before any considerable, i.e. > 0.5°C, decline of body temperature. Decrease in peripheral vasomotor tone was inferred from the increases in comb and unfeathered leg temperatures after clonidine; however, comb temperature only increased when it was initially low, that is when ambient temperature was at or near the lower end of the thermoneutral range. Once the fall in body temperature with clonidine was maximal, heat-conservation replaced heat-loss mechanisms, viz. feather erection, wing droop and squatting (by squatting, fowls reduced heat loss by one-third compared with standing fowls; Deighton & Hutchinson, 1940).

Heat-loss mechanisms were also activated by relatively large doses of catecholamines. Thus noradrenaline infused into the hypothalamus or IIIrd ventricle induced tachypnoea and wing abduction; intraventricular isoprenaline or dopamine also evoked tachypnoea (Marley & Nisticò, 1972). The fall in body temperature following noradrenaline was accompanied by elevation or depression of comb temperature, according to whether the latter was initially low or high; elevation of comb temperature was not sustained, as after clonidine. Increase in skin temperature of the unfeathered feet follows application of noradrenaline to the hypothalamus of adult pigeons or young chicks (Hissa & Rautenberg, 1974; Marley & Stephenson, 1975). As with clonidine, once the fall in body temperature was marked, heat-conservation replaced heat-loss mechanisms.

Augmented heat-loss could therefore partly account for the fall in body temperature after clonidine. Additionally, CO_2 elimination was 50% reduced, implying decreased metabolism which would also lower body temperature. The decline in CO_2 elimination, as with the heat-loss mechanisms, was well developed before body temperature had fallen to any extent. This decline resembled that induced in chicks below thermoneutrality by noradrenaline infused into the hypothalamus, a decline attributed to inhibition of utilization of non-esterified fatty acids (Marley & Stephenson, 1975); clonidine may well act similarly. Fowls utilize lipids rather than carbohydrates for heat production (Freeman, 1967). The fall in body temperature with clonidine was enhanced and prolonged when the fowl was at an ambient temperature below thermoneutrality, a feature noted with intravenous or intrahypothalamic infusion of catecholamines (Allen & Marley, 1967; Allen, Garg & Marley, 1970; Marley & Stephenson, 1975).

Intraventricular phentolamine attenuated the hypothermic effects of centrally applied clonidine. The dose of phentolamine was crucial, since doses two to four times those used for the antagonism experiments provoked marked and even fatal hyperthermia. Other antagonists administered intraventricularly, viz propranolol, haloperidol, methysergide and atropine, did not attenuate the hypothermic effects of clonidine.

Feldberg & Saxena (1971) tested the effect of α-adrenoceptor antagonists given intraventricularly on the assumption that noradrenaline was released continuously in the hypothalamus to control body temperature. Compatible with this idea, in cats, which respond like fowls with a fall in body temperature to centrally applied noradrenaline, hyperthermia developed after intraventricular phenoxybenzamine or phentolamine (Feldberg & Saxena, 1971). Since noradrenaline was also hypothermic in fowls at or below thermoneutrality, it was scarcely surprising that blockade of its receptors elevated body temperature. Intraventricular atropine also elevated body temperature, a finding more difficult to interpret since acetylcholine and other cholinomimetics infused intraventricularly or into the hypothalamus of chickens at thermoneutrality, lacked temperature effects (Marley & Seller, 1972, 1974a, b); a hypothermic action of these substances did emerge when chickens were tested below thermoneutrality (Stephenson & Marley, unpublished). It seems therefore that metabolic processes in the fowl maintain a high body temperature, exceeding that in mammals, which is kept within physiological limits at thermoneutrality by hypothalamic release of noradrenaline and possibly of acetylcholine. Since intraventricular methysergide, in doses effective against centrally applied 5-hydroxytryptamine, did not affect body temperature it would appear that 5-hydroxytryptamine release into the fowls' hypothalamus had little to do with temperature regulation at thermoneutrality.

The interaction between clonidine and dexamphetamine was of interest. Thus, behavioural and electrocortical arousal induced by dexamphetamine in adult fowls were replaced by sleep following clonidine infused intraventricularly, into the hypothalamus or intravenously. Similarly, arousal elicited by dexamphetamine was replaced by sleep following catecholamines given intravenously in young chicks (Key & Marley, 1962; Dewhurst & Marley, 1965), and intraventricularly or into the hypothalamus of adult fowls (Marley & Nisticò, 1975). These results, together with the evidence that clonidine and noradrenaline act on central α-adrenoceptors, are difficult to reconcile with the notion that central noradrenaline release mediates arousal, whether drug-induced or not.

There are possible explanations for these findings. First, intraventricular clonidine and noradrenaline may affect different populations of central α-adrenoceptors from those involved in arousal associated with amphetamine-induced noradrenaline release. While this is likely, it still does not explain replacement of dexamphetamine-induced arousal by sleep following intravenous noradrenaline in young chicks or intravenous clonidine in young or adult fowls, since the overall brain distributions of the three compounds after administration were likely to be similar.

Clonidine and exogenous noradrenaline may have different effects from noradrenaline liberated endogenously. The evidence is against this for certain parts of the brain, since the soporific and hypothermic effects of noradrenaline infused into the IIIrd cerebral ventricle or hypothalamus were markedly potentiated by amine oxidase inhibition (Schain, 1961; Schmidt & Fähse, 1964; Marley & Stephenson, 1970; Marley & Nisticò, 1972), potentiation implying that noradrenaline was taken up into neurones, escaped deamination and was then released, i.e. endogenously. Moreover, dexamphetamine given intraventricularly to cats (Gaddum & Vogt, 1956) or infused into the hypothalamus of fowls (Marley & Nisticò, 1975) induced sleep, that is noradrenaline released endogenously by dexamphetamine, mimicked the effects of exogenous noradrenaline.

Extending this argument to other parts of the brain, it could be that the excitant action of dexamphetamine was due to the effects of noradrenaline released in the brain-stem preponderating over those of noradrenaline liberated elsewhere in the brain, particularly the hypothalamus (Marley, 1973). This is feasible, since noradrenaline perfused bilaterally into the pontine and mesencephalic reticular formation of feline encéphale isolé preparations, evoked behavioural and electrocortical arousal (Key, 1975), whereas noradrenaline given intraventricularly or into the hypothalamus induced sleep (Feldberg & Sherwood, 1954; Myers, 1964).

One of us (E.M.) is indebted to the Royal Society for a travel Study Award. Our thanks are due to Dr J.D. Stephenson for measuring CO_2 elimination, to the Bethlem Royal and Maudsley Hospitals Research Funds for support and to Boehringer-Ingelheim for supplying the clonidine.

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(Received November 27, 1974.
Revised July 1, 1975.)

Br. J. Pharmac. (1975), 55,475-486

EFFECTS OF DOPAMINE ON SINUS RATE AND VENTRICULAR CONTRACTILE FORCE OF THE DOG HEART *In vitro* AND *in vivo*

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1 Experiments were carried out on dog isolated papillary muscle and sinus node preparations perfused with arterial blood from a donor dog. The chronotropic and inotropic effects of dopamine were analysed by using reserpine, desmethylimipramine (DMI), cocaine and phenoxybenzamine, and the relative chronotropic and inotropic effects defined, the results being compared with those for noradrenaline (NA).

2 The effects of dopamine administered intra-arterially into the isolated preparations were reduced by pretreatment of animals with reserpine both in the papillary muscle and sinus node. The chronotropic effects, however, were affected less by pretreatment with reserpine than were the inotropic effects.

3 Desmethylimipramine (DMI) reduced the inotropic and chronotropic effects of dopamine, and enhanced the effects of NA and nerve stimulation; the chronotropic effects of the amines were less affected than the inotropic effects.

4 Cocaine enhanced considerably the inotropic and chronotropic effects of NA, and decreased the inotropic but not the chronotropic effect of dopamine.

5 Phenoxybenzamine enhanced the inotropic effects of dopamine, NA and nerve stimulation, but did not affect the chronotropic effects of the amines.

6 When dopamine (1 to 300 μg/kg) was administered intravenously to the donor dog, it increased preferentially the contractile force of the ventricular myocardium with a comparatively small change of the sinus rate in the isolated preparations as well as in the heart *in vivo*. NA (0.1 to 10 μg/kg) caused effects similar to those of dopamine. The maximal inotropic responses to these catecholamines were reached with lower doses than the chronotropic ones.

7 It is concluded that both the positive inotropic and the positive chronotropic responses to dopamine are mediated partially by a direct and partially by an indirect stimulant effect on β-adrenoceptors in the dog heart. The present results suggest that the difference in activity of dopamine and NA between the ventricular myocardium and the sinus node may be ascribed to the unequal innervation with adrenergic nerve fibres of the atrium and the ventricle (Furnival, Linden & Snow, 1971). The sinus node which is densely innervated by adrenergic nerve fibres may inactivate noradrenaline and dopamine more effectively than the ventricular myocardium through the uptake into the nerve and thereby be less sensitive to the exogenous catecholamines.

Introduction

Dopamine has been shown to have both direct and indirect sympathomimetic actions on the heart in various species. In human subjects and in the dog, it increases myocardial contractile force at doses which have little effect on heart rate (Maxwell, Rowe, Castillo, Clifford, Afonso & Crumpton, 1960; Holmes & Fowler, 1962; Horwitz, Fox & Goldberg, 1962; McDonald & Goldberg, 1963). Goldberg (1972) concluded that dopamine differs from other catecholamines in this respect. However, Furnival, Linden & Snow (1971) have shown that noradrenaline caused a much greater inotropic change than isoprenaline for a given chronotropic change in the denervated dog heart. Tuttle (1970) has suggested that the positive chronotropic effect of dopamine is due to direct stimulation of β-adrenoceptors, whereas the positive inotropic effect is largely indirect. This assumption was based on the finding that in the anaesthetized dog, following vagotomy, the positive inotropic effect of dopamine, but not the positive chronotropic effect could be abolished with desmethylimipramine (DMI). This latter observation is at variance with the finding that the positive chronotropic response to dopamine is reduced by reserpine in the dog (Bejrablaya, Burn & Walker, 1958), the guinea-pig (Tsai, Langer & Trendelenburg, 1967), rabbit (Lee & Yoo, 1964) and cat (Farmer, 1966).

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The present study was undertaken to elucidate further the mechanism of action of dopamine on the myocardium by the use of reserpine, DMI, cocaine and phenoxybenzamine, and the results were compared with those for noradrenaline (NA) on the isolated, blood-perfused papillary muscle (Endoh & Hashimoto, 1970a, b) and sinus node (Kubota & Hashimoto, 1973) preparations of the dog. Comparisons were made between the *in vitro* effects of dopamine and NA and the effects of the amines on myocardial contractile force and heart rate *in vivo* in the donor dog.

Methods

Mongrel dogs of either sex weighing between 7 and 14 kg were anaesthetized with sodium pentobarbitone (30 mg/kg i.v.). Each animal was given an intravenous dose of heparin (500 u/kg) and the heart removed. The ventricular septum together with the anterior papillary muscle of the right ventricle, and the right atrium containing the sinus node, were excised separately and placed in cold Tyrode solution gassed with 95% O_2 and 5% CO_2. The anterior septal artery and the sinus node artery respectively were cannulated and were perfused with arterial blood from a donor dog at a constant perfusion pressure of 100 mmHg using a rotating pump (Watson-Marlow Ltd., type MHRE 200). The venous effluent from the preparations was collected in a reservoir and returned to the donor dog through the jugular vein. The donor dog was anaesthetized with sodium pentobarbitone (30 mg/kg i.v.); heparin (500 u/kg) was given intravenously immediately before the perfusion began and subsequent doses of heparin (200 u/kg) given at intervals of 1 hour. The temperature of the tissues was maintained at 37-38° C. Details of the experimental procedures used in perfusion of the papillary muscle and the sinus node have been described previously (Endoh & Hashimoto, 1970a, b; Kubota & Hashimoto, 1973). The papillary muscle was driven at a rate of 120 beats/min with square wave pulses of 5 ms duration and a stimulus strength of 1 V (about 1.5 times the threshold voltage). The developed tension of the papillary muscle was measured isometrically with a force displacement transducer (Statham gold cell with microscale accessory Model UL5). The rate of the isolated sinus node was recorded by means of a cardiotachograph (Grass 7P4D) triggered by the electrogram of the sinus node region, using a unipolar silver electrode. The maximal rate of developed tension (dT/dt) and perfusion pressure of the preparation, together with the blood pressure and heart rate of the donor dog, were also constantly recorded and displayed on an inkwriting oscillograph (Grass Model 7B polygraph). The blood flow of the preparations was measured by collecting blood in a graduated cylinder or by means of an electromagnetic flow meter (Nycotron, Model 375).

Drug solutions, in a volume of 0.01-0.03 ml, were injected into the rubber tubes connected to the arterial cannula of each of the isolated preparations. Injections were made over a period of 4 s with a microsyringe (Jintan Terumo Co.). Cocaine was infused at a rate of 0.01 ml/min into the base of the Y piece connecting the outflow from the donor dog to the two preparations.

Release of autonomic transmitters in response to field stimulation (FS) of the papillary muscle (Endoh & Hashimoto, 1970b) was obtained by raising the voltage of the driving pulse of the muscle from 1 V to 5 V or 10 V for 15 or 30 seconds. Perivascular nerve stimulation (PNS) of the papillary muscle (Endoh, Hashimoto & Kimura, 1972) was performed by applying a pulse of 1 ms duration and 3-20 V at 10-20 Hz for 15 or 30 s through bipolar silver electrodes attached around the septal artery.

In seven experiments, a left thoracotomy was performed in the donor dog. A Walton-Brodie strain-gauge arch was sutured to the surface of the right ventricle for the recording of myocardial contractile force, and the cervical vagi divided. Drugs were administered via a cannulated femoral vein. Chronotropic and inotropic responses to dopamine and NA were recorded from the intact heart of the donor dog and the isolated preparations, simultaneously. Inotropic effects were expressed either in absolute values (g in the papillary muscle and mm in the donor dog) or in percentage changes of the active tension.

Drugs used in these experiments were dopamine hydrochloride, (−)-noradrenaline bitartrate, tyramine hydrochloride, prindolol (Visken), reserpine, desmethylimipramine hydrochloride (DMI, Geigy), cocaine hydrochloride and phenoxybenzamine.

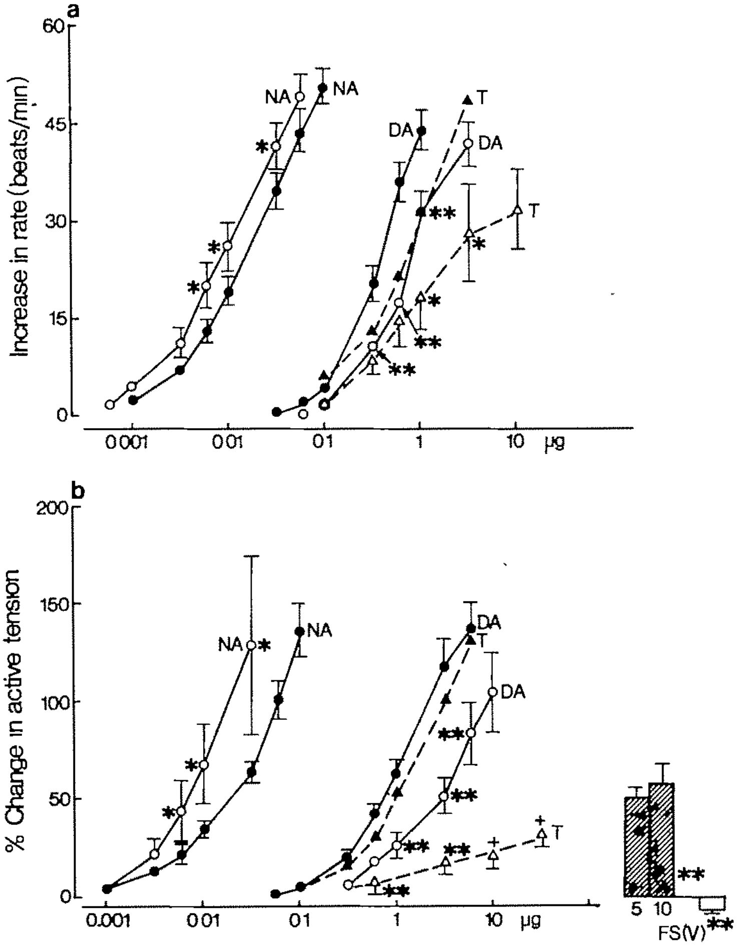

Figure 1 Effects of reserpine on mean responses to noradrenaline (NA), dopamine (DA), tyramine (T) and field stimulation (FS) in the dog. (a) Isolated sinus node preparation and (b) isolated papillary muscle. The sympathomimetic amines were directly injected into the sinus node or papillary muscle artery. Since the blood flow of the papillary muscle preparation is about 5 times that of the sinus node preparations, the concentration of the amines in the sinus node preparation is higher than that in the papillary muscle preparation. Closed symbols and hatched columns: preparations from untreated animals (n = 15 to 27 in NA, DA and FS; n = 6 in T); open symbols and open columns: preparations from reserpine-pretreated animals (n = 6); vertical bars show s.e. mean. $^*P < 0.05$ compared with responses to the corresponding doses of amines and to nerve stimulation in preparations from untreated animals; $^{**}P < 0.01$; $^+P < 0.001$ compared with the response to 6 µg of tyramine.

DMI and phenoxybenzamine were dissolved in 30% ethanol solution; close arterial injection of 0.01 ml of 30% ethanol solution caused small negative inotropic and negative chronotropic effects lasting for only 1 to 2 minutes.

Reserpine was given in a dose of 3 mg/kg subcutaneously to 9 animals 24 h before the experiment. Six of these animals were used to provide the isolated preparations, 3 were donor animals.

Doses of prindolol, phenoxybenzamine and reserpine refer to the base; doses of the other drugs refer to the corresponding salt.

All experimental values are given as means with s.e. mean. Statistical comparisons were performed by means of Student's t test. The test for paired

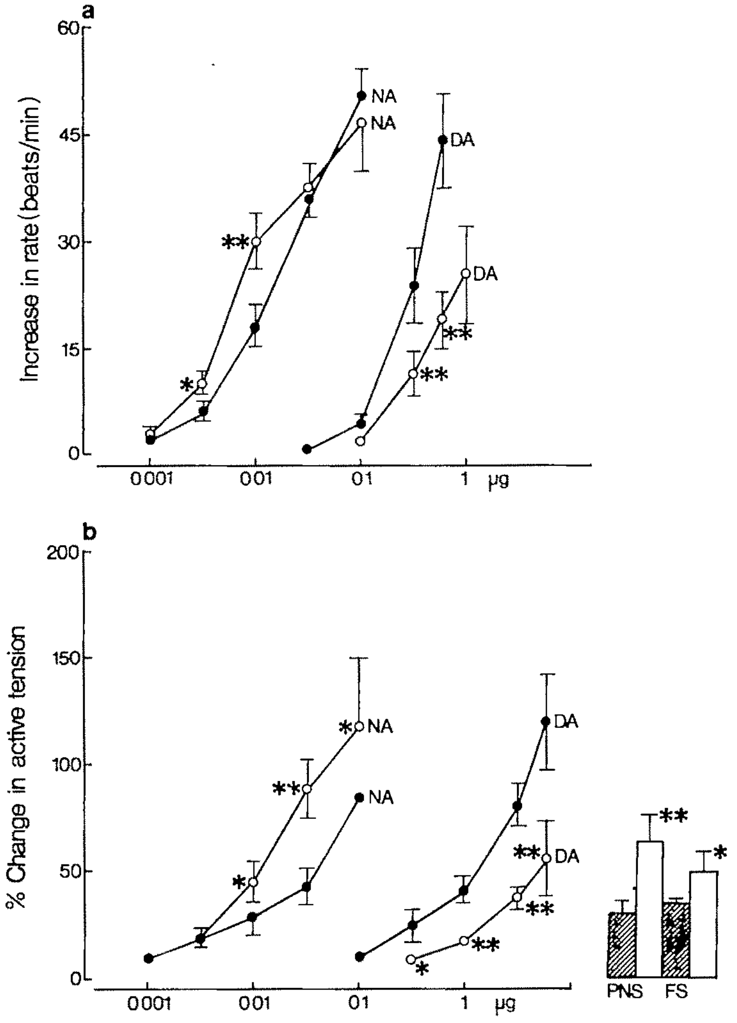

Figure 2 Effects of desmethylimipramine (DMI) on mean responses to noradrenaline (NA), dopamine (DA), perivascular nerve stimulation (PNS) and field stimulation (FS) in dog (a) isolated sinus node preparation and (b) isolated papillary muscle. Closed symbols and hatched columns: control responses; open symbols and open columns: following administration of DMI 100 µg; $n = 3$ to 6; vertical bars show s.e. mean. $^*P < 0.05$ compared with responses to the corresponding doses of NA and dopamine, and to nerve stimulation in the absence of DMI-treatment; $^{**}P < 0.01$.

comparison was used when applicable. A P value of less than 0.05 was considered to be significant.

Results

Effects of noradrenaline and dopamine on sinus rate and active tension in the isolated preparations

NA and dopamine injected directly into sinus node or papillary muscle artery increased the rate of contraction of the sinus node preparation and increased active tension in the papillary muscle preparation over a wide range of doses. The responses were dose-dependent; the dose-response curve for the two catechlamines were parallel; NA was approximately 50 times more potent than dopamine on both preparations, when they were compared in molar doses (Figure 1). Since the effects of NA and dopamine on the maximum rate of developed tension (dT/dt) in the papillary muscle were similar to the effects on active

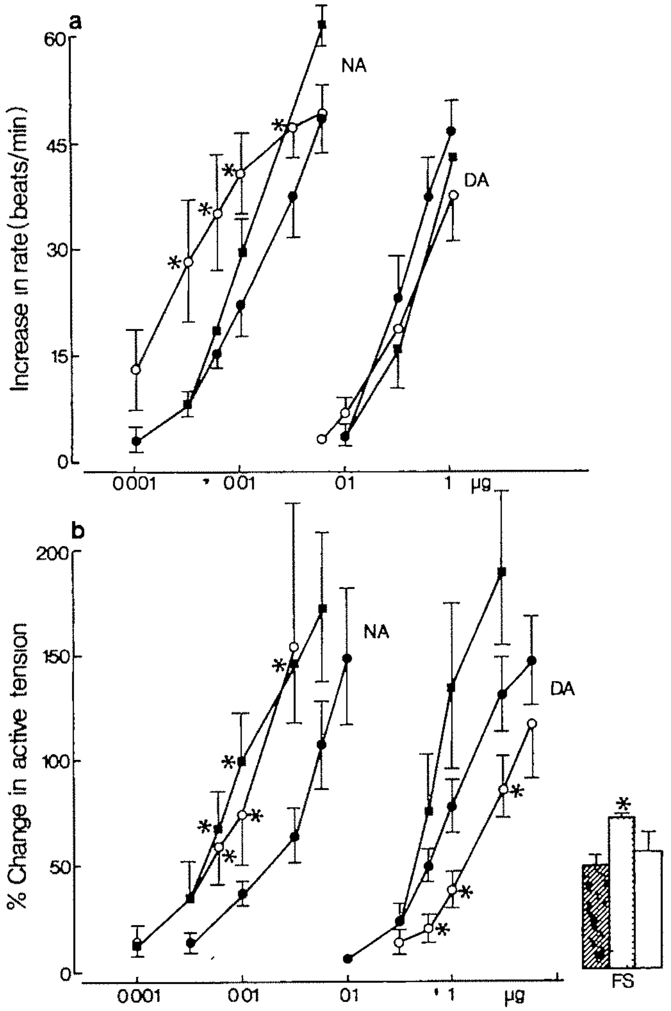

Figure 3 Effects of cocaine on mean responses to noradrenaline (NA), dopamine (DA) and field stimulation (FS) in dog (a) isolated sinus node preparation and (b) isolated papillary muscle. (●) and hatched column: control responses; (■) and dotted column: during infusion of cocaine 3 µg/min; (○) and open column, during infusion of cocaine 10 µg/min; $n = 3$ to 8 in NA and dopamine; $n = 2$ to 7 in field stimulation; vertical bars show s.e. mean. $^*P < 0.05$ compared with responses to the corresponding doses of NA and DA, and to nerve stimulation before infusion of cocaine.

tension, only the effects on active tension will be described.

The responses to dopamine (0.3 to 1.0 µg) as well as noradrenaline (0.01 to 0.1 µg) and field stimulation could be abolished by the β-adrenoceptor blocking agent prindolol, 10 µg, administered to each preparation. Prindolol has previously been shown to exert a long-lasting block of β-adrenoceptors with little stimulant or depressant actions in a study of seven β-blocking agents tested in the present preparations (Hashimoto, Ohkuda, Chiba & Taira, 1969; Hashimoto, Endoh, Tamura & Taira, 1970).

Effect of reserpine

The dose-response curves obtained in tissues from animals pretreated with reserpine showed a shift to the left in the case of NA whereas the dose-response curves for dopamine and tyramine were displaced to the right (Figure 1). The effect of pretreatment with reserpine on responses to NA, dopamine and tyramine were more marked on the papillary muscle than on the sinus node. The dose-response curves in the control and reserpine-pretreated preparations were compared in the part where curves are substantially parallel, i.e. increase

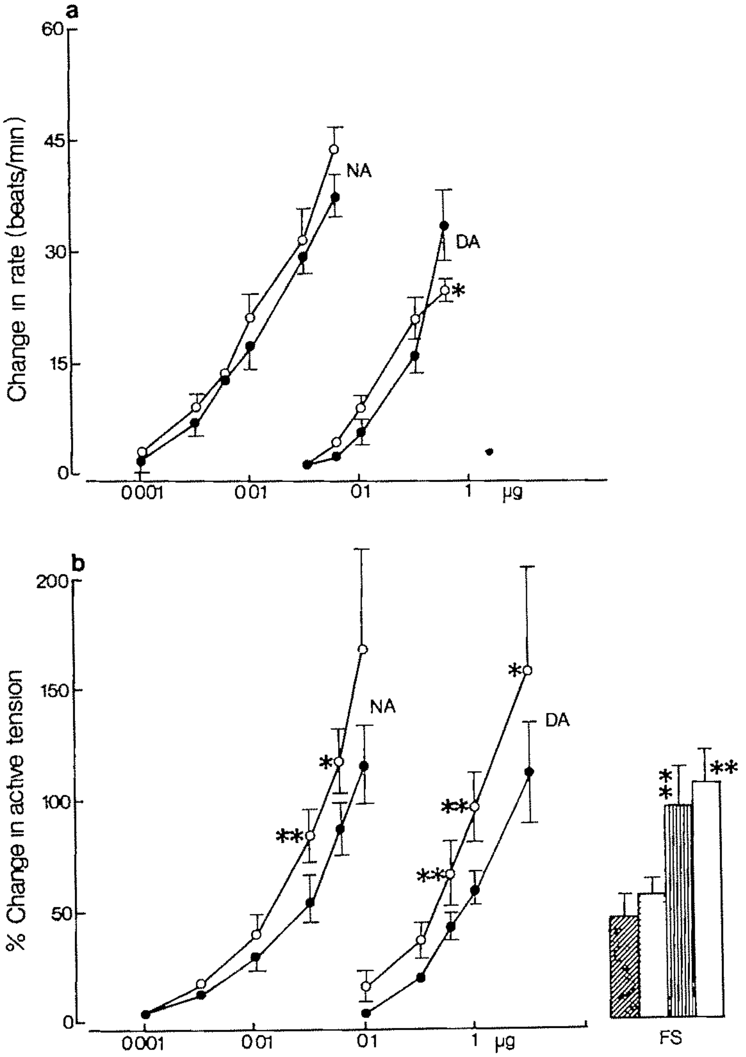

Figure 4 Effects of phenoxybenzamine on mean responses to noradrenaline (NA), dopamine (DA) and field stimulation (FS) in dog (a) isolated sinus node preparation and (b) isolated papillary muscle (●) and diagonally hatched column: control responses; dotted column: responses following phenoxybenzamine 100 µg; vertically hatched column: following phenoxybenzamine 300 µg; (○) and open column: following phenoxybenzamine 1000 µg; $n = 4$ to 8; vertical bars show s.e. mean. $^*P < 0.05$ compared with responses to the corresponding doses of NA and dopamine, and to nerve stimulation before the administration of phenoxybenzamine; $^{**}P < 0.02$.

of 25 beats/min in the sinus node and 80% increase of active tension in the papillary muscle preparation. The curves for NA were shifted to the left by a factor of 2.2 in the sinus node and 3.2 in the papillary muscle; the shift of curves for dopamine to the right was 2.6 in the sinus node and 4.0 in the papillary muscle. Reserpine completely abolished the positive inotropic response to field stimulation: field stimulation in these preparations induced a reduction in active tension, a response which could be blocked by atropine (Endoh & Hashimoto, 1970b).

Effects of desmethylimipramine (DMI) and cocaine

Close-arterial injections of 100 µg of DMI to the sinus node or papillary muscle caused transient negative followed by positive chronotropic or inotropic responses lasting from 5 to 10 min,

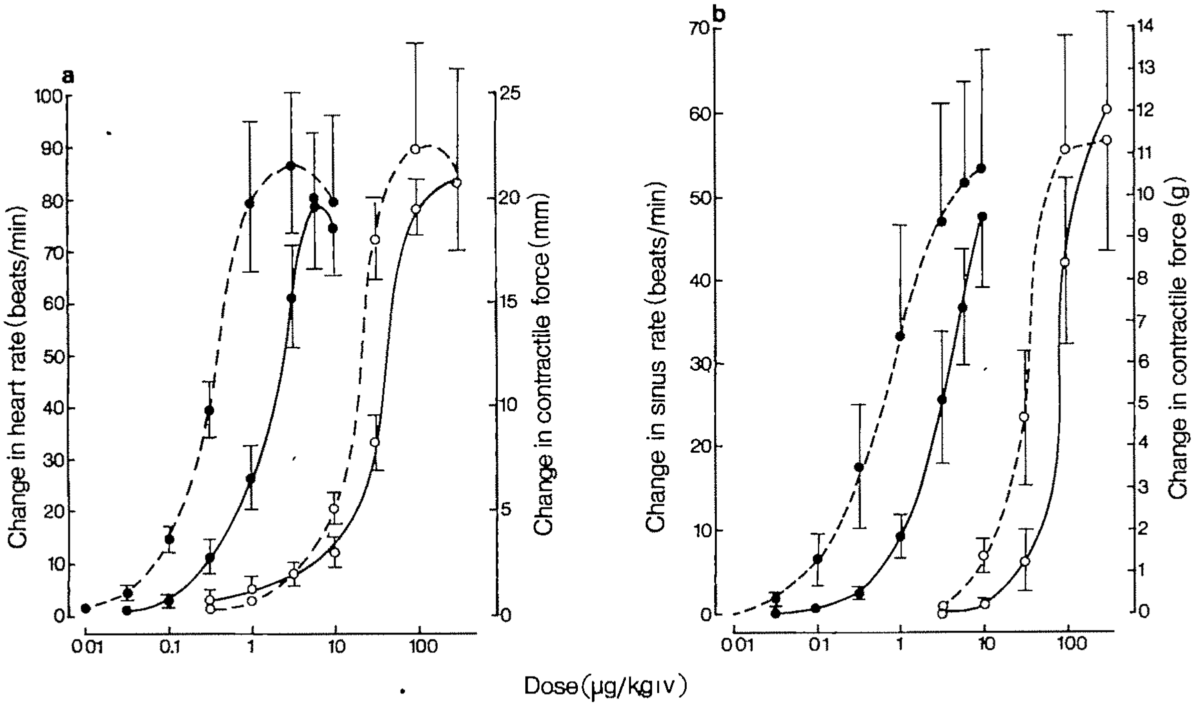

Figure 5 Effects of noradrenaline (NA) (●) and dopamine (o) administered intravenously into the vagotomized donor dog on the sinus rate (solid lines) and the contractile force (dotted lines). (a) Donor dog ($n = 7$); (b) isolated preparations ($n = 4$). Vertical bars show s.e. mean.

which could be abolished by pindolol. Subsequent responses to NA in both preparations and positive inotropic responses to perivascular nerve stimulation and field stimulation were enhanced; in contrast, responses to dopamine were smaller (Figure 2). The shift of the dose-response curves for NA to the left and for dopamine to the right occurred to the same degree, i.e. by a factor of 2 in the sinus node and 4 in the papillary muscle.

Cocaine alone had little effect on either preparation at the infusion rates used (3 and 10 µg/minute). Responses to dopamine, NA and nerve stimulation were obtained before and again from 10 min after beginning an infusion of cocaine. Mean results are shown in Figure 3. Responses to NA were considerably enhanced during infusion of both doses of cocaine: the shift of the dose-response curves for NA to the left during infusion of the high dose of cocaine was by a factor of about 4 in both preparations. The inotropic response to dopamine was enhanced slightly but not significantly during infusion of the low dose of cocaine and decreased significantly at the higher dose: the dose-response curve for dopamine was shifted to the right by a factor of 2.5. In the sinus node preparation, however, response to dopamine was not significantly changed during infusion of cocaine at either rate.

Effect of phenoxybenzamine

Phenoxybenzamine in doses of 100, 300 and 1000 µg, injected close-arterially into the sinus node or papillary muscle artery, caused positive chronotropic or inotropic responses lasting between 10 and 30 minutes. After the rate of contraction of the sinus node preparation and the developed tension of the papillary muscle had returned to the control level, the effects of dopamine, NA and nerve stimulation were examined in each preparation. The positive inotropic effect of field stimulation was enhanced dose-dependently by phenoxybenzamine (Figure 4). The positive inotropic responses to dopamine as well as to NA were significantly enhanced after treatment with 1000 µg of phenoxybenzamine: the shift of curves for both amines was about 2-fold. In contrast, the positive chronotropic effects of NA and dopamine were affected less than inotropic effects of the amines. The chronotropic response to a high dose of dopamine (0.1 µg) was significantly decreased (Figure 4).

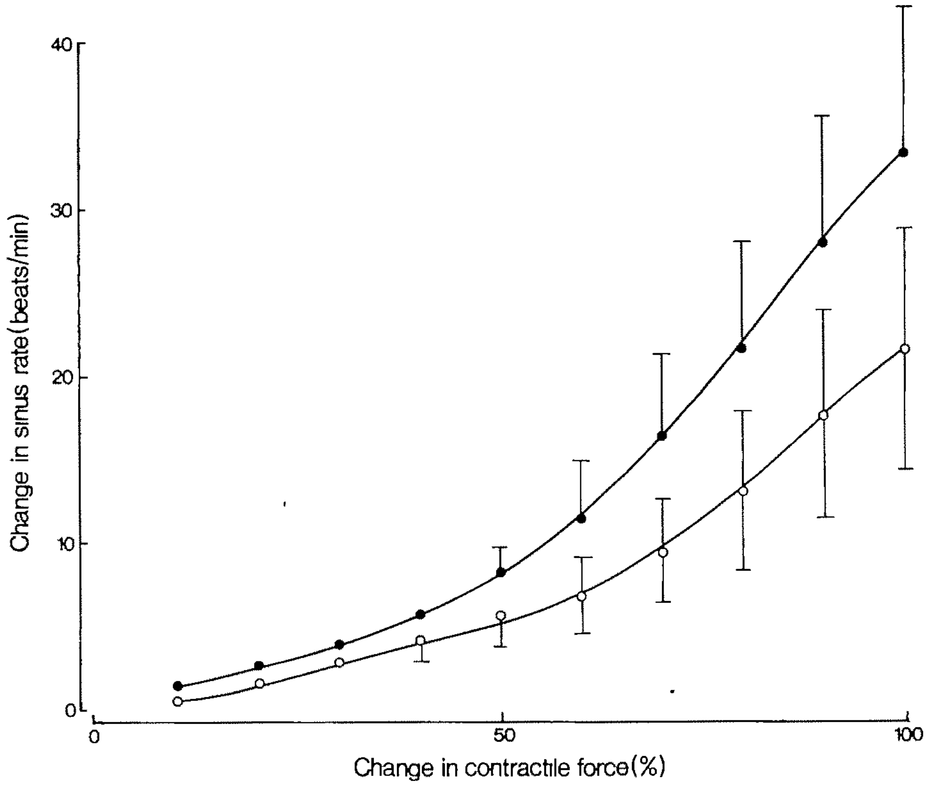

Figure 6 Inotropic-chronotropic relationships of noradrenaline (●) (NA) and dopamine (○) in the isolated preparations ($n = 4$). Increases in the rate (ordinate in beats/min) of the isolated sinus node preparation in response to intravenous doses of NA and dopamine which caused equal positive inotropic effects in the papillary muscle preparations are estimated. Vertical bars show s.e. mean.

Effects of intravenous administration of dopamine and noradrenaline

The mean changes in ventricular contractile force and heart rate in response to a wide range of doses of dopamine and NA administered intravenously to the donor dog are shown in Figure 5. The threshold doses for inducing the positive inotropic responses were lower than those for eliciting the positive chronotropic responses. In the donor dogs, 0.1 μg/kg of NA increased ventricular contractile force by 39.2 ± 9.6% ($P < 0.001$; calcu-

Table 1 The ED_{50} and ED_{100} for noradrenaline (NA) and dopamine (DA) administered intravenously to the donor dog (μg/kg)

		Donor dog			*Isolated preparations*		
		PIA	*PCA*	*PCA/PIA dose-ratio*	*PIA*	*PCA*	*PCA/PIA dose-ratio*
NA	ED_{50}	0.36	1.6	4.5	0.63	> 2.9	> 4.6
	ED_{100}	3	6		10	—	
DA	ED_{50}	22	39	1.8	35	> 74	> 2.1
	ED_{100}	100	300		300	—	

PIA: positive inotropic action; PCA: positive chronotropic action; ED_{100} and ED_{50}: doses required for eliciting the maximal and half of maximal response, respectively; PCA/PIA dose-ratio was calculated from doses which induce the half of maximal responses.

lated as a percentage change from the value in mm in Figure 5). A significant increase in heart rate (10 ± 3 beats/min; $P < 0.025$) was first obtained following 0.3 μg/kg of NA. In the isolated papillary muscle, 0.3 μg/kg of NA increased developed tension by 18.0 ± 4.9% ($P < 0.02$); the rate of the isolated sinus node was first significantly increased with 1.0 μg/kg of NA by 9 ± 2 beats/min ($P < 0.02$). Dopamine, 30 μg/kg, increased developed tension in the papillary muscle significantly by 46.7 ± 12.1% ($P < 0.02$); the rate of the isolated sinus node was first significantly increased with 100 μg/kg of dopamine by 35 ± 11 beats/min ($P < 0.05$).

The dose-response curves for NA and dopamine in the contractile force and sinus rate are substantially parallel both in the donor dog (Figure 5a) and in the isolated preparations (Figure 5b). The maximal inotropic responses to NA and dopamine were reached both in the isolated papillary muscle and the heart *in vivo*, while the maximal chronotropic responses were achieved only in the donor dog but not in the isolated sinus node. The ED_{50} and the ED_{100} for NA and dopamine are given in Table 1. NA as well as dopamine showed greater activity in the ventricular myocardium than in the sinus node *in vivo* and *in vitro*: NA and dopamine were more potent in the inotropic action by factors of 4.5 and 1.8 or more, respectively, than in the chronotropic action.

In order to analyse the relative chronotropic and inotropic effects of dopamine and NA more precisely, increases in the rate of the isolated sinus node preparation in response to intravenous doses of NA and dopamine which caused equal positive inotropic effects in the papillary muscle were estimated from the dose-response curve in each experiment. Figure 6 shows the inotropic-chronotropic relationships of NA and dopamine in the isolated preparations. The sinus node preparation was less sensitive to both catecholamines: when the developed tension in the papillary muscle was increased by 50% with NA and dopamine, respectively, increases in the rate of contraction of the sinus node preparation were less than 10 beats/minute.

Discussion

Mechanism of action of dopamine

In the *in vitro* experiments described here, responses to dopamine were inhibited in both the sinus node and papillary muscle preparations, and responses to NA potentiated in tissues obtained from animals pretreated with reserpine. Responses of the sinus node to dopamine and to NA were affected less by reserpine-pretreatment than were those of the papillary muscle. Also the chronotropic response to tyramine was only partially inhibited by reserpine-pretreatment, whereas the positive inotropic response of the papillary muscle to field stimulation was completely blocked and the positive inotropic response to tyramine was greatly reduced by reserpine-pretreatment. Tuttle (1970) showed in the anaesthetized, vagotomized dog that the chronotropic response to dopamine was also more resistant to DMI than that of ventricular contractile force; the present findings in the dog isolated heart preparations that the shift to the right caused by DMI in the dose-response curve for dopamine in the papillary muscle was twice that in the sinus node are consistent with the results of Tuttle. He concluded that the chronotropic effect of dopamine was due to a direct action on β-adrenoceptors and that much of the inotropic effect depended on NA release. However, in the present study not only the inhibitory effect of DMI on the response to dopamine but also the enhancing effect of DMI on the response to NA was less in the sinus node than in the papillary muscle preparation. These observations indicate that the sinus node preparation is more resistant to the blocking effect of DMI on the neuronal uptake of the amines (Iversen, 1967) as well as to depletion of NA by reserpine: the effect of dopamine on the sinus node is fundamentally not different from that on the papillary muscle. Thus, findings with reserpine and DMI support the view (Bejrablaya *et al.*, 1958; Lee & Yoo, 1964; Farmer, 1966; Tsai *et al.*, 1967; Chiba, Hashimoto & Hashimoto, 1973; Neuvonen & Westermann, 1973) that both the positive inotropic and the positive chronotropic responses to dopamine are mediated partially by a direct and partially by an indirect stimulant effect on β-adrenoceptors.

The infusion of cocaine (10 μg/min) greatly enhanced the positive chronotropic and inotropic effects of NA, while the positive chronotropic effect of dopamine was not changed significantly and the positive inotropic effect of dopamine was reduced significantly but to a lesser extent compared with the enhancement of responses to NA. Trendelenburg (1959, 1961), using the nictitating membrane of the spinal cat, observed that the injection of a small amount of cocaine (0.2 mg/kg) increased the contractile response to tyramine and NA, and assumed that the enhancement was due to a pronounced sensitization by cocaine of the effector organ to endogenously liberated NA and of a slight reduction of the releasing effectivity of tyramine. The present effects of cocaine (10 μg/min) on responses to dopamine and NA, and of the low dose (3 μg/min) to enhance the positive inotropic response to dopamine, are compatible with findings of Trendelenburg (1959, 1961). cocaine may cause supersensitization of the postsynaptic effector cells of the sinus node and the papillary muscle.

Phenoxybenzamine enhanced the positive inotropic effects of dopamine and NA to the same degree in the papillary muscle; this effect of phenoxybenzamine may be ascribed to its blocking action on the extraneuronal uptake of the amines (Iversen, 1967). On the other hand, the positive chronotropic effects of the amines are not affected by phenoxybenzamine. It is assumed that the extraneuronal uptake of amines is less in the sinus node than in the papillary muscle, because of anatomical differences between these tissues *viz.* the group of sinus node cells are perfused with a single main sinus node artery in the dog (Hashimoto, Tanaka, Hirata & Chiba, 1967), while the papillary muscle is perfused with many small branches of the septal artery (Endoh & Hashimoto, 1970b). The reason for inhibition by phenoxybenzamine of the positive chronotropic effect of a high dose of dopamine is unknown: it is not explained by the effect of phenoxybenzamine on the neuronal uptake of amines, because the effect of NA was not affected.

The relative chronotropic and inotropic effects of dopamine

Since it is evident that the chronotropic response to drugs in the heart *in situ* is modified by extracardiac factors, and furthermore that drug effects on ventricular contractile force are affected by changes in heart rate (Furnival, Linden & Snow, 1970), it is difficult to state exactly the inotropic-chronotropic relationship of drugs in the heart *in situ.* On the other hand, the isolated sinus node and papillary muscle preparations were perfused with the same concentration of drugs, and were not affected by extra-cardiac factors or frequency-dependent effects of drugs on contraction. It has been shown that the isolated preparations reflected well the blood level of the drugs, catecholamines and ouabain, in the donor dog (Hashimoto, Kimura & Kubota, 1973). Furthermore, if the donor dog is maintained carefully, the response to catecholamines injected close-arterially remains constant for several hours (Hashimoto *et al.,* 1970; Endoh, Kimura & Hashimoto, 1972): the amount of drugs required to cause responses in the isolated preparations is so small that re-circulation in the donor dog is negligible.

Dopamine given by the intravenous route to the donor dog caused marked increases in myocardial contractile force with comparatively little change in heart rate *in vivo* and in the isolated preparations. These results are in agreement with the findings reported for man and the dog (Maxwell, Rowe, Castillo, Clifford, Afonso & Crumpton, 1960; Holmes & Fowler, 1962; Horwitz *et al.,* 1962, McDonald & Goldberg, 1963). This differential effect on heart rate and contractile force has been considered to be specific for dopamine (Goldberg, 1972), although there has been no satisfactory comparison between the cardiac effects of dopamine and other catecholamines. Recently, Furnival *et al.* (1971) have shown that NA also produced less rise in heart rate than does isoprenaline for a similar increase in contractile force in the denervated dog heart. In the present experiments, the effects of dopamine on heart rate and contractile force were compared with those of NA. Intravenous administration of NA resulted in very similar effects to those observed with dopamine. The dose-response curves obtained for NA were parallel to those for dopamine. NA was approximately 50 times more potent than dopamine both in its effects on contractile force and heart rate. A similar potency ratio was found in the *in vitro* experiments. These experiments clearly show that the effects of dopamine on heart rate and contractile force are not specific for dopamine, but are shared by NA.

The question arises as to the reason for the difference in activity of NA and dopamine between the ventricular myocardium and the sinus node. It is known that the uptake mechanism of amines plays an important role in inactivating the exogenous and endogenous NA: Iversen (1967) has shown that there are two different mechanisms for uptake of NA, i.e. uptake into the nerve endings (uptake$_1$) and uptake into tissues other than nerve (uptake$_2$). On the other hand, the atrium, especially the right atrium, contains more transmitter substance, NA, than the ventricular myocardium in various species of animals (Muscholl, 1959; Cervoni, Kirpekar & Schwab, 1966). In the dog, Shore, Cohn, Highman & Maling (1958) have found that the sinus node region contains 2.3 μg/g NA, while the right papillary muscle contains 1.5 μg/gram. Angelakos, Fuxe & Torchiana (1963) have further shown histochemically that the catecholamines were located in nerve structures and that a high density of fluorescent fibre bundles were found in the sinus node region of the rabbit. Since it is commonly considered that the indirectly acting sympathomimetic amines are probably taken into adrenergic neurones by a common amine transfer system (Starke, 1972), the sinus node which is densely innervated by adrenergic fibres may have a larger capacity to inactivate the exogenous NA and dopamine by taking up these amines more actively into the nerve and thereby show a lower sensitivity to these amines than the ventricular myocardium. This view has already been put forward by Furnival *et al.*.(1971) on the basis of their findings that NA produced less rise in heart rate than did isoprenaline for a similar increase in contractile force, while the relative chronotropic and inotropic effect of NA equalled that of isoprenaline after the blockade of neuronal uptake by cocaine in the denervated dog.

The excellent technical assistance of Mr Rudolf Meier is gratefully acknowledged. The author wishes to thank Professor H.J. Schumann and Dr B Clark for useful comments on the manuscript.

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(Received January 8, 1975.
Revised May 15, 1975.)

Br. J. Pharmac. (1975), 55, 487-495

SOME PHARMACOLOGICAL PROPERTIES OF THE CREMASTER MUSCLE OF THE GUINEA-PIG

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1 The tension developed in the guinea-pig cremaster was recorded during spontaneous activity and electrical stimulation. Spontaneous rhythmic contraction was observed in many preparations, particularly in the tip of the cremaster. These contractions were very slow, lasting about 20 s and occurring at about 2 min intervals, but different preparations varied greatly. Twitches produced by electrical stimulation were similar to those in other skeletal muscles, being reduced by (+)-tubocurarine and abolished by tetrodotoxin.

2 A slow contraction could be initiated by electrical stimulation using a pulse longer than 10 ms in spontaneously active preparations and in some quiescent preparations. Spontaneous and evoked slow contractions were not suppressed by tetrodotoxin, but were selectively abolished by verapamil. Histamine increased the basal tension and generated spontaneous contractions in quiescent preparations. Hypertonic solutions also had excitatory effects.

3 Contractions produced by acetylcholine and carbachol were blocked by atropine. Those produced by adrenaline and noradrenaline were stronger than those due to histamine, acetylcholine and carbachol and were abolished by an α-adrenoceptor blocking agent, phentolamine. In the preparations in which the slow contraction was not observed, histamine, acetylcholine or adrenaline had very little effect.

4 It is suggested that the cremaster muscle consists of striated muscle together with some smooth muscle having properties similar to that in the vas deferens.

Introduction

The cremaster muscle has been considered as a striated muscle. In the rat this muscle is composed of two layers which are extensions of the internal oblique and transversus abdominis muscles (Greene, 1955; Grant, 1966). The aim of the present experiments was to study the mechanical and pharmacological properties of the cremaster in an attempt to elucidate possible differences from typical striated muscles.

Methods

Male guinea-pigs weighing 250-350 g were stunned and bled. The sac of cremaster muscle containing the testis was excised. The testis was gently pulled out from the sac, turning the inside of the sac out. The cremaster was mounted under slight tension (about 0.2 g) in an organ bath (3 ml), through which a physiological salt solution (containing (mM): NaCl 120.7, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 15.5, glucose 11.5) flowed at a rate of about 1 ml/minute. The bath was maintained at 36°C and was aerated with mixture of 3% CO_2 and 97% O_2. In some experiments, the cremaster was divided into two, one half containing the neck of the sac and the other half containing the tip of the sac. Tension was recorded by means of a strain gauge. A pen recorder was used only for the slow mechanical responses; when fast twitch responses to electrical stimulation were studied, the response was displayed on a cathode-ray oscilloscope and recorded on film. Electrical stimulation was applied transversely through an assembly of Ag-AgCl electrodes similar to that described by Hill (1949).

Drugs were injected into flowing solution near the inlet of the organ bath to give the stated concentration in the organ bath. This concentration was maintained only briefly after injection and was thereafter progressively diluted by the solution flowing through.

Drugs used were (+)-tubocurarine chloride, tetrodotoxin citrate (Sankyo), verapamil, histamine hydrochloride, acetylcholine chloride,

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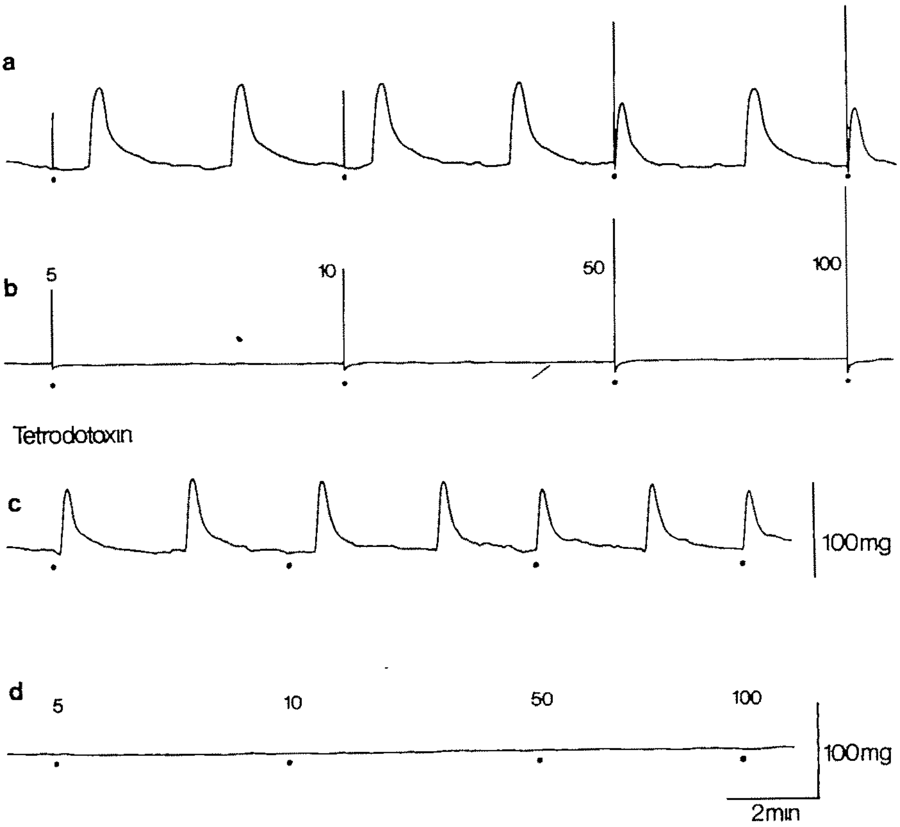

Figure 1 Effects of tetrodotoxin (0.5 μg/ml) on evoked twitches (stimulation is shown by dots and numbers indicate duration of pulse in ms), and on spontaneous and evoked slow contractions. Mechanical responses were simultaneously recorded isometrically from two preparations, divided into tip (a, c) and neck (b,d) of the cremaster but mounted in the same organ bath. Tetrodotoxin blocked the fast twitches but not the slow contractions. Only the tip produced the slow contractions.

carbachol, atropine sulphate (−)-adrenaline hydrochloride, (−)-noradrenaline bitartrate and phentolamine mesylate. The concentrations of the drugs refer to their salts.

Results

Spontaneous activity and mechanical responses to electrical stimulation

In many preparations, spontaneous slow rhythmic contractions were observed. When the tip of the cremaster was used, these contractions were observed in 80% of preparations ($n = 20$). Spontaneous contractions were not so common (20%, $n = 20$) in the neck of the cremaster. The frequency of rhythmic contractions varied greatly in different preparations. The range was 0.006-0.093 Hz ($n = 15$) and the frequency often decreased or the spontaneous activity stopped completely during the course of experiments. The average time to reach peak tension was 7.5 s (range: 4-12; $n = 11$) and to reach 50% relaxation was 6.8 s (range: 3.5-10; $n = 11$). The maximum tension of the slow contraction was very small (usually about 100 mg) compared with that of the fast twitch (about 2 g).

Figure 1 shows the mechanical responses recorded from the tip (a and c) and neck (b and d) of the cremaster. The tip showed spontaneous activity but the neck was quiescent. Single electrical pulses shorter than 10 ms evoked fast twitches only, but a pulse longer than 50 ms evoked fast twitches followed by a slow contraction which was similar to the spontaneous contraction in the tip of the cremaster (a). No

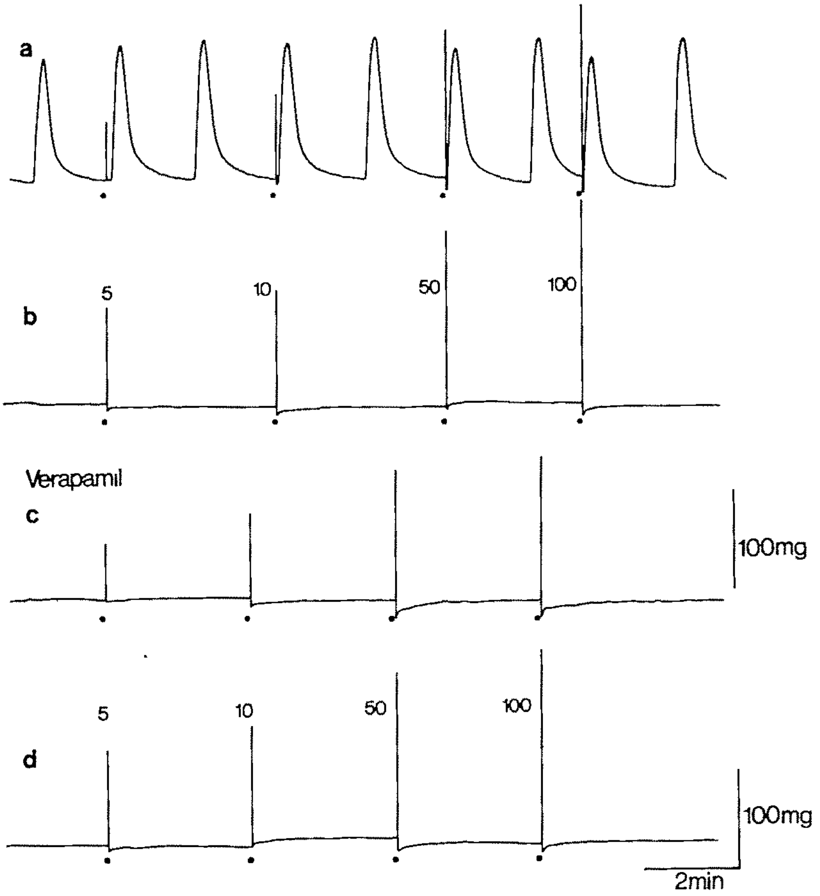

Figure 2 Effects of verapamil (10 μg/ml) on spontaneous and evoked responses simultaneously recorded from the tip (a, c) and neck (b, d) of cremaster. Dots and numbers indicate stimulation and pulse duration in milliseconds. Verapamil abolished the slow contractions only.

slow contraction was evoked in the neck of the cremaster (b). Tetrodotoxin (0.5 μg/ml) blocked the fast twitch responses, but not the spontaneous and evoked slow contractions (c and d) which were also little affected by (+)-tubocurarine (10 μg/ml).

In contrast to the effect of tetrodotoxin, verapamil (10 μg/ml) selectively abolished the spontaneous contractions and evoked slow contractions (Figure 2c) whereas the twitches were little changed. The blocking effect of verapamil was reversible.

Table 1. Twitch tension produced by single maximal shocks in three muscles of the guinea-pig

	Time to peak (ms)			*Time to half relaxation (ms)*		
Pulse duration (ms)	0.5	1	5	0.5	1	5
Extensor digitorum longus	10.8	11.2	17.1	12.7	12.5	9.4
Cremaster	19.7	21.9	28.5	17.9	26.0	32.4
Soleus	47.8	54.0	59.7	51.5	58.5	48.0

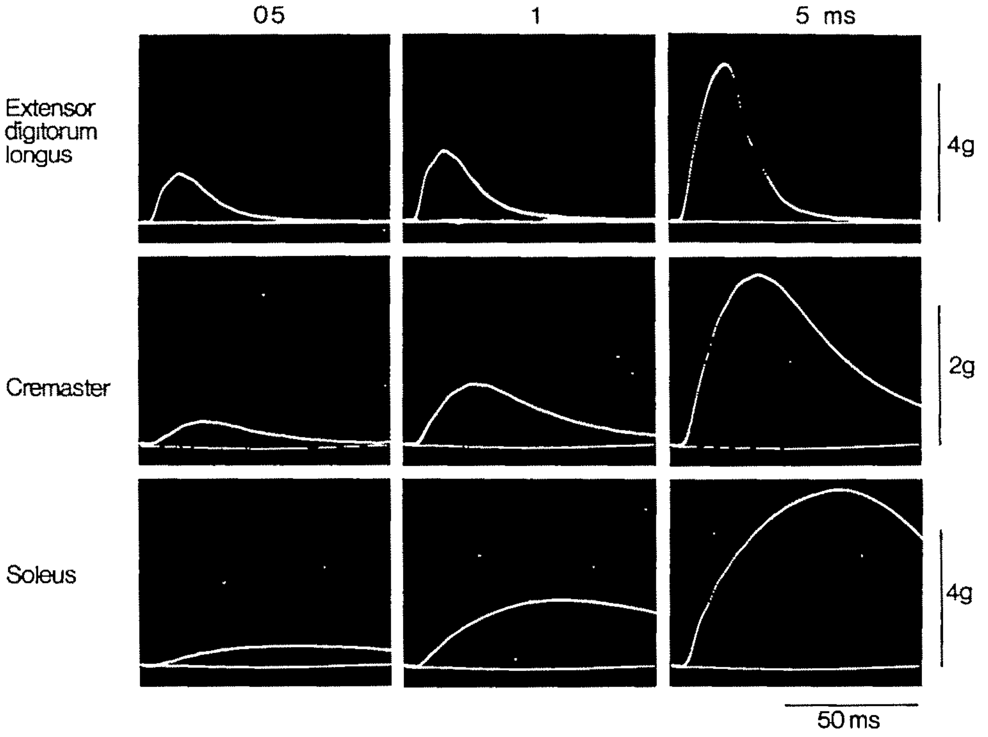

Figure 3 Isometric tension during twitches of extensor digitorum longus (top), cremaster (middle) and soleus (bottom), elicited by electrical stimulation of three different pulse durations (0.5, 1 and 5 milliseconds).

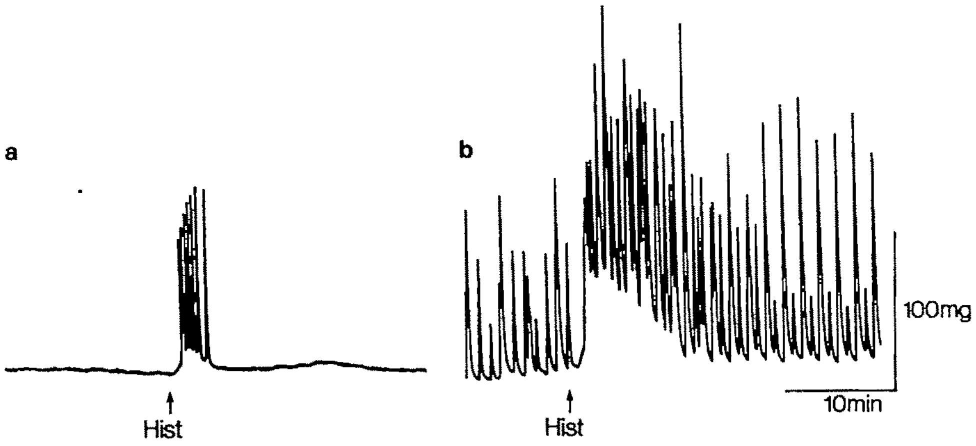

Figure 4 Effects of histamine (Hist), 1 μg/ml (a), and 5 μg/ml (b) in two different, inactive (a) and spontaneously active (b) preparations of the tip of the cremaster.

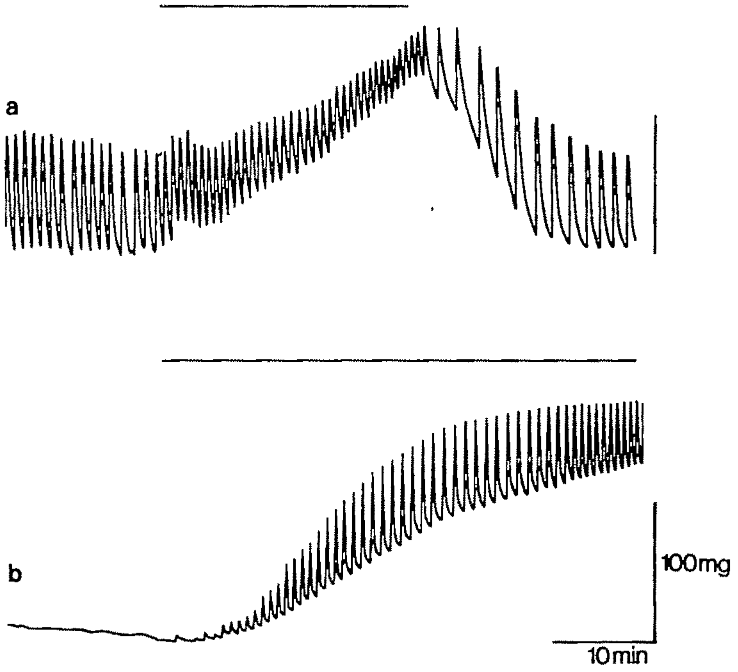

Figure 5 Effects of hyperosmotic solution made by the addition of 5 g sucrose to 100 ml of physiological salt solution. Perfusion of hyperosmotic solution is indicated by horizontal bars. Two different preparations, (a) having spontaneous activity and (b) no activity.

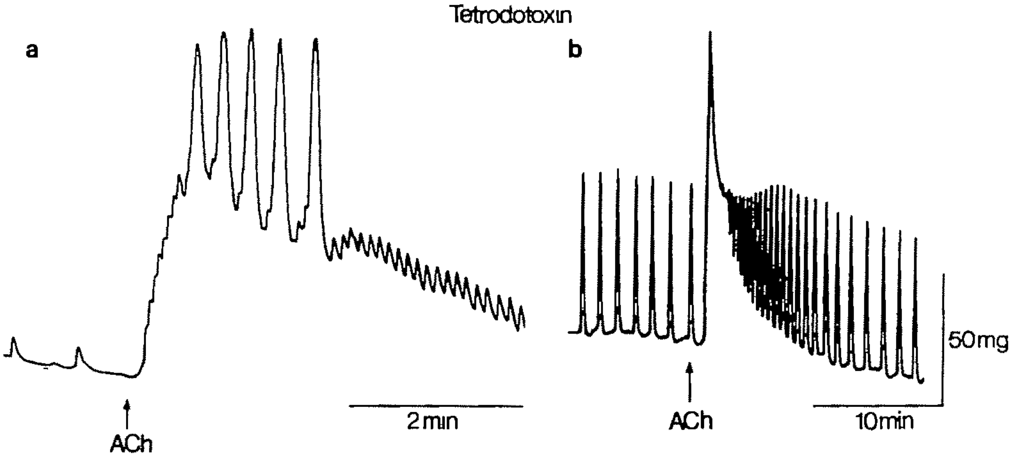

Figure 6 Effects of acetylcholine (ACh), 10 μg/ml, on two different preparations of whole cremaster muscle. The acetylcholine response in (b) was recorded in the presence of tetrodotoxin (0.5 μg/ml).

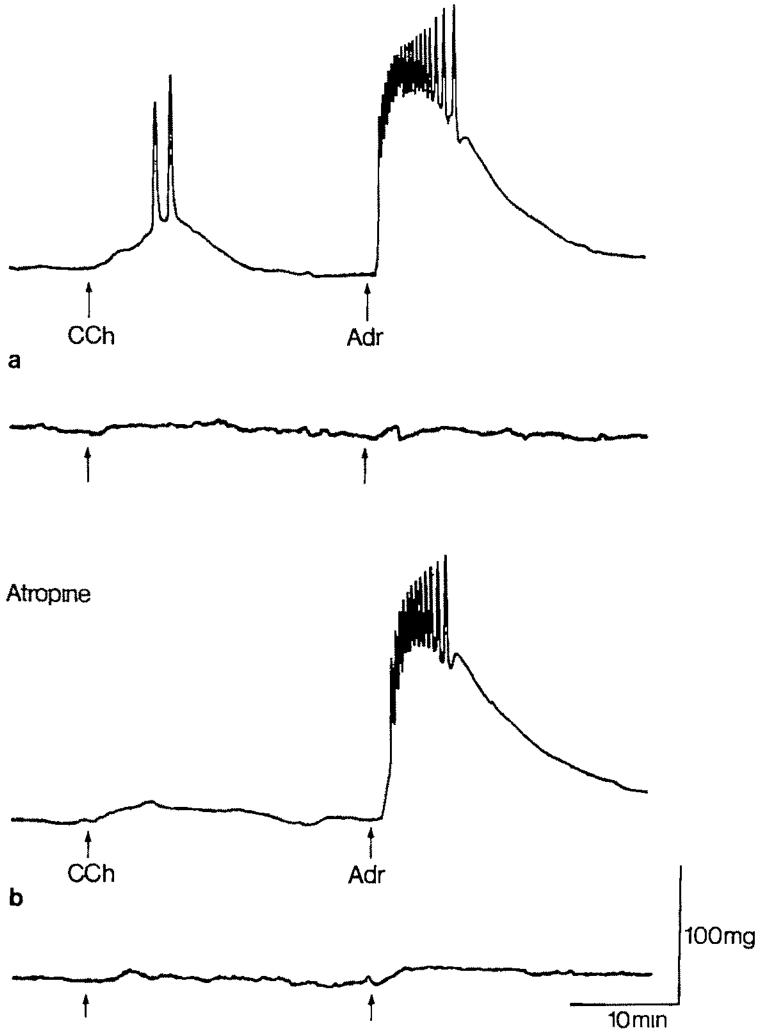

Figure 7 Responses to carbachol (CCh), 1 μg/ml, and adrenaline (Adr), 1 μg/ml, (a) before and (b) after atropine (1 μg/ml). In both (a) and (b) the upper trace recorded from the tip, and the lower trace from the neck of the cremaster.

Twitches produced in the cremaster by single maximal shocks at three different pulse durations were compared with those of the extensor digitorum longus and soleus muscles (Figure 3). The average times to reach the peak tension and half relaxation are summarized in Table 1. The time course of twitch tension of the cremaster was slower than the extensor digitorum longus, which is a typical fast muscle, but faster than the soleus, which is a typical slow muscle. The values for the extensor muscle and the soleus were slightly faster than those previously reported by Tashiro (1973). The difference may be due partly to the higher temperature (36° C instead of 33° C), and partly to the smaller size of the animal (250-350 g instead of 300-400 g) in the present experiments.

Twitch tensions were reduced by (+)-tubocurarine (10 μg/ml), the reduction being 80% with a pulse of 0.5 ms, 39% with 1 ms and 14% with 5 ms pulse. It is likely that the muscle fibre is directly stimulated by long current pulses. Tetrodotoxin (0.5 μg/ml) completely blocked all twitches.

Histamine (1 to 5 μg/ml) had a stimulant action

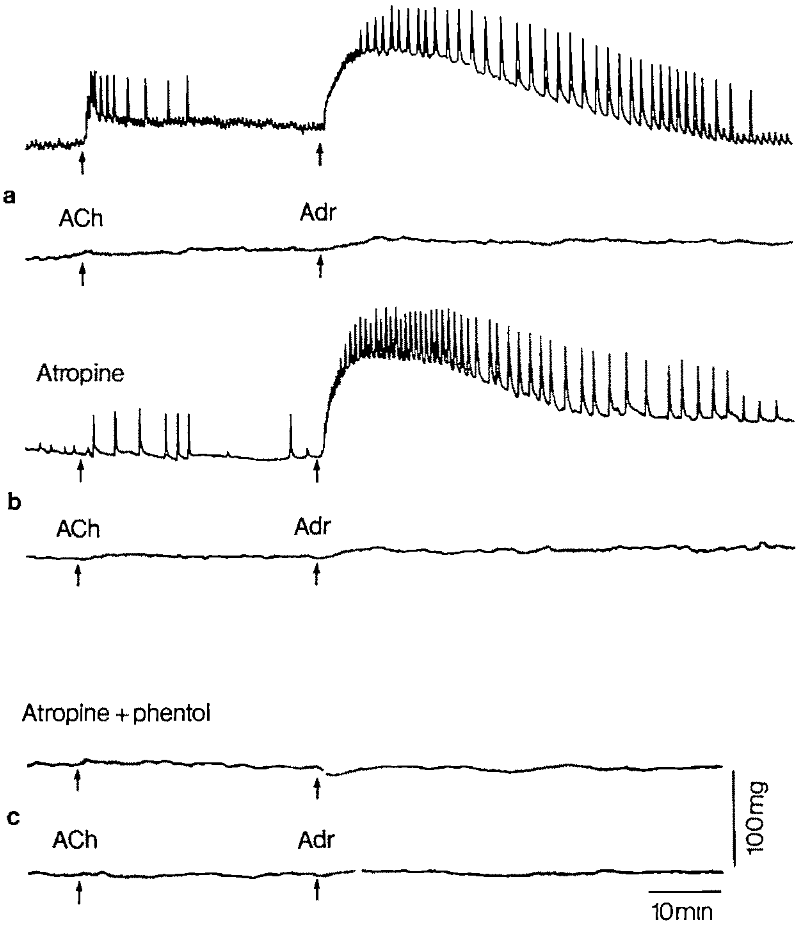

Figure 8 Responses to (a) acetylcholine (ACh), 5 μg/ml, and adrenaline (Adr), 5 μg/ml as modified by (b) atropine, 1 μg/ml, (c) atropine, 1 μg/ml and phentolamine, 1 μg/ml. In (a), (b) and (c) the upper trace is from the tip and the lower trace from the neck of the cremaster.

on the tip, but it usually failed to give a response in the neck of the cremaster. Histamine initiated rhythmic slow contractions in a quiescent preparation (Figure 4a), and it raised the basal tension and increased the frequency of slow contractions in a spontaneously active preparation (Figure 4b).

Figure 5 shows effects of solutions made hyperosmotic by adding sucrose to the salt solution. The hyperosmotic solution increased the basal tension and the frequency of spontaneous contractions (a); it often produced spontaneous slow contractions in quiescent preparations (b).

Effects of parasympathomimetic and sympathomimetic drugs and antagonists

Acetylcholine (1-10 μg/ml) produced a rise in basal tension and increased the frequency of spontaneous contractions. In 20% of preparations ($n = 20$) which had no spontaneous activity or showed small contractions, acetylcholine produced spontaneous activity or increased the magnitude of contractions in the whole cremaster preparation, as shown in Figure 6a. These effects were not abolished by tetrodotoxin (1 μg/ml, Figure 6b). Carbachol produced responses similar to those with acetylcholine (Figure 7a). The responses caused by acetylcholine or carbachol were suppressed by atropine (1 μg/ml, Figure 7b). In the neck of the cremaster, which did not exhibit the slow spontaneous activity, acetylcholine and carbachol failed to increase the basal tension or a slow contraction (Figure 7).

Adrenaline (1 μg/ml) also had stimulant actions, causing an increase in the basal tension and the generation of spontaneous contraction in the tip of the cremaster (Figure 7). When spontaneous activity existed, increases in magnitude and in frequency of the slow contractions were usually produced by adrenaline. These effects of adrenaline were not affected by atropine (Figure 7b) or tetrodotoxin. The effect of noradrenaline (1 μg/ml) was similar to that of adrenaline.

Figure 8 shows responses to acetylcholine (5 μg/ml) and adrenaline (5 μg/ml). Only the tip of the cremaster (top traces) responded to the drugs (a). The cremaster was always more sensitive to adrenaline than to acetylcholine or carbachol. The response to acetylcholine was suppressed by atropine (1 μg/ml), but that to adrenaline was not (b). The contraction produced by adrenaline was abolished by the α-adrenoceptor blocking drug phentolamine (1 μg/ml), and instead there was often a small relaxation, as shown in the upper trace of Figure 8c.

The preparation shown in Figure 8 produced small spontaneous contractions at the beginning of the experiment, but the spontaneous activity gradually disappeared. In other preparations in which the spontaneous activity persisted, phentolamine abolished the response to adrenaline but did not block spontaneous activity.

Discussion

The speed of a twitch response to electrical stimulation in the cremaster muscle is slower than in the extensor digitorum longus, but faster than in the soleus. The twitch is blocked by tetrodotoxin which is known to abolish the action potential in most nerves and striated muscles (Kao, 1966; Kuriyama, Osa & Toida, 1966). These properties of the cremaster are those of skeletal muscle.

The slow contractions of the cremaster are quite different from the twitch, and are readily stimulated by adrenaline, which also raises the tone of the tissue. It is possible that this peculiarity is attributable to smooth muscle contained in the cremaster. This could also account for the anaphylactic response observed in the guinea-pig cremaster (Alonso-deFlorida, Ninomiya & Paz, 1972). It may be argued that the property of the cremaster is similar to that of denervated striated muscle, since it is known that the anaphylactic response can be observed in the guinea-pig diaphragm after denervation (Alonso-deFlorida, del Castillo, González & Sánchez, 1965; Alonso-deFlorida, del Castillo, García & Gijón, 1968) and that adrenaline produces contracture in the denervated rat diaphragm (Bhoola & Schachter, 1961). The simplest hypothesis is that the cremaster, particularly at its tip, consists of both striated muscle and smooth muscle.

Supporting evidence for the possibility that the slow contraction is generated in smooth muscle is (1) that the speed of slow contraction is too slow to be a response of striated muscle, (2) that tetrodotoxin, which does not affect the action potential in smooth muscle (Kuriyama *et al.*, 1966; Bülbring & Tomita, 1967), has no effect on the slow contraction, (3) that verapamil which blocks the action potential in smooth muscles (Golenhofen & Lammel, 1972) selectively suppresses the slow contraction and (4) that effects of histamine, cholinomimetic and sympathomimetic agents are similar to those observed in smooth muscles such as the vas deferens, which receive adrenergic innervation.

In a preliminary histological study smooth muscle has been seen in close connection with the blood vessels. It seems that this muscle is not part of the wall of the blood vessels, but further studies are necessary to come to a firm conclusion. The density of blood vessels of a medium size (10-50 μm) is much higher in the tip of the cremaster than the neck of the muscle. This corresponds with the observation that the slow contraction is recorded mainly from the tip of the muscle. It has been shown that hyperosmotic solution produces vasodilatation in the vascular bed in skeletal muscle and suppression of spontaneous activity in the rat portal vein (Mellander, 1960; Mellander, Johansson, Gray, Jonsson, Lundvall & Ljung, 1967; Johansson & Jonsson, 1968). However, the spontaneous activity in the cremaster is increased in hyperosmotic solution. Although topical application of adrenaline results in constriction of the blood vessels in the rat cremaster, intravenous application of adrenaline and acetylcholine dilates the blood vessels (Grant, 1964). If the spontaneous activity in the cremaster is generated in the blood vessels, their properties seem to be different from those of the blood vessels in striated muscles. Further experiments are necessary to see whether the slow contractions observed in the present experiments are due to the presence of smooth muscle.

I am grateful to Professor T. Tomita and Dr T. Gotow, Fukuoka University, for their valuable help during this work. My thanks are also due to Professor N. Toida for the privilege of working in his Department. The author is a recipient of the scholarship from the National Council of Science and Technology (Conacyt) in Mexico and the Ministry of Education in Japan.

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(Received January 13, 1975.
Revised May 16, 1975)

Br. J. Pharmac. (1975), 55, 497-501

FURAZOLIDONE-PETHIDINE INTERACTION IN RABBITS

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1 The intravenous injection of pethidine in rabbits pretreated with furazolidone administered orally but not systemically resulted in severe interaction and fatal hyperpyrexia.

2 Treatment with *p*-chlorophenylalanine, chloropromazine or cyproheptadine protected the rabbits against the furazolidone-pethidine interaction, while α-methyl-*p*-tyrosine was ineffective.

3 5-Hydroxytryptophan produced a fatal hyperpyrexia in furazolidone pretreated rabbits.

4 Pretreatment of rabbits with 1,1,1-trichloro-2, 2-bis(*p*-chlorophenyl)ethane (DDT) accelerated and enhanced the furazolidone-pethidine interaction, while oxytetracycline pretreatment completely prevented the interaction.

5 It is concluded that furazolidone-pethidine interaction might depend mainly on potentiation of the effects of 5-hydroxytryptamine in the CNS and that the transformation of furazolidone into an active monoamine oxidase inhibitor metabolite might occur mainly in the gut microflora in the gut lumen.

Introduction

Furazolidone ((N-5-nitro-2-furfurylidene) 3-amino-2-oxazolidinone) is an antibacterial agent recommended for its local action in the intestinal tract and vagina (Ponce de Leon, 1957; Schneierson & Bryer, 1959). When administered to rats and man, furazolidone causes monoamine oxidase (MAO) inhibition (Stern, Hollifield, Wilk & Buzard, 1967; Pettinger, Soyangco & Oates, 1968), although it has no effect on this enzyme *in vitro*. A metabolite of furazolidone is thought to be responsible for the MAO inhibition (Stern *et al.*, 1967).

Since furazolidone is used extensively in tropical countries for the treatment of gastroenteritis, it was thought of interest to study its possible interactions with pethidine in rabbits. It is known that pethidine causes excitation and hyperpyrexia in rabbits pretreated with MAO inhibitors (Nymark & Nielsen, 1963; Loveless & Maxwell, 1965; Penn & Rogers, 1971; Fahim, Ismail & Osman, 1972; Sinclair, 1972). The symptoms in rabbits resemble those that occur in man, thus the rabbit seems to be a suitable model to use in this investigation. It was also the purpose of this work to investigate whether this interaction can be modified by pretreatment with drugs which interfere with the synthesis or antagonize the brain monoamines. In addition, experiments were carried out to see whether the interaction can be modified by pretreatment of rabbits with a broad spectrum antibiotic such as oxytetracycline or with an inducer of drug metabolizing enzymes such as 1,1,1-trichloro-2, 2,-bis(*p*-chlorophenyl)-ethane (DDT) to find out where biotransformation of furazolidone to the active MAO inhibitor takes place.

Methods

Rabbits of local strains and of either sex weighing between 1.5 and 2.5 kg were used. Furazolidone was administered orally by a stomach tube in a dose of 50 mg/kg daily for four successive days. Control rabbits received 10 ml of 0.9% w/v NaCl solution (saline) orally. On the fifth day, the animals were restrained in stocks and rectal temperatures were recorded at 15 min intervals with electrical thermocouples (Ellab electric universal thermometer, type T.E.3) inserted 5-7 cm into the rectum. When the temperature of a rabbit had been stable for 30 min, pethidine (5 mg/kg) was slowly injected via a marginal ear vein. The experiments were carried out at 21-22° C.

The effect of pethidine was also tested in furazolidone-treated animals which had also received one of the following pretreatments:

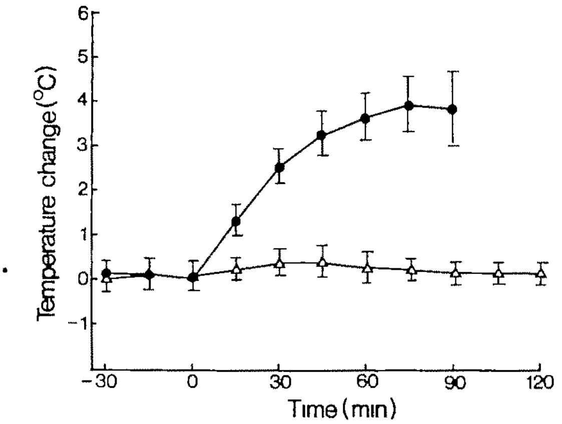

Figure 1 Effect of pethidine on the rectal temperature of rabbits pretreated with furazolidone. Pethidine (5 mg/kg i.v.) was injected at time zero in one group of five rabbits (●), the other group of four rabbits was pretreated with furazolidone and injected with saline (△). Doses are given in the text. Vertical lines indicate s.e. mean.

chloropromazine hydrochloride or cyproheptadine hydrochloride (5 mg/kg, i.v.) 30 min before pethidine; *p*-chlorophenylalanine (PCPA) (125 mg/kg i.p.) 66, 42, and 18 h before pethidine; α-methyl-*p*-tyrosine (80 mg/kg i.p.) 48, 36, 24 and 12 h before pethidine; oxytetracycline (50 mg/kg i.p.) twice daily and also once daily orally (50 mg/kg) for seven days before pethidine; DDT (50 mg/kg i.p.) injected daily for four successive days before furazolidone pretreatment. 5-Hydroxytryptophan (5-HTP) (60 mg/kg) was injected intravenously.

Drugs

The following drugs were used: *p*-chlorophenylalanine (Sigma), chloropromazine (May & Baker), cyproheptadine (Merck, Sharp & Dohme), furazolidone (Eaton), 5-hydroxytryptophan (Sigma), α-methyl-*p*-tyrosine (Sigma), oxytetracycline (Pfizer), pethidine (Evans), and 1,1,1-trichloro-2, 2-bis(*p*-chlorophenyl)ethane (DDT) (B.D.H.). The doses given in the text refer to the salts, whenever applicable.

Results

Rabbits treated with furazolidone, pethidine, *p*-chlorophenylalanine, α-methyl-*p*-tyrosine, cyproheptadine, 5-HTP, oxytetracycline, or DDT did not show any significant change in their body temperature when compared with saline-treated control animals.

Furazolidone-pethidine interaction

Pethidine injection produced motor restlessness, shivering like tremor, hyperexcitability, tachypnoea and hyperpyrexia in furazolidone pretreated rabbits (Figure 1). The mean increase in temperature was 3.9°C, 75 min after pethidine injection and 3 out of 5 rabbits died in hyperpyrexia. Control rabbits receiving saline orally when injected with pethidine did not show any significant rise in their temperature. Also rabbits pretreated with furazolidone and injected with saline did not show any significant change in their temperature. In some rabbits furazolidone was injected subcutaneously in a dose of 50 mg/kg daily for four successive days and this resulted in a very mild interaction when pethidine was injected on the fifth day.

Pretreatment with p-chlorophenylalanine, cyproheptadine, chloropromazine, and α-methyl-p-tyrosine. Interaction of furazolidone with 5-hydroxytryptophan

The furazolidone-pethidine hyperpyrexic interaction was completely prevented by PCPA treatment and none of the rabbits pretreated with PCPA died in hyperpyrexia. Similarly the injection of either cyproheptadine or chloropromazine in furazolidone pretreated rabbits 30 min before pethidine injection protected the rabbits against the fatal hyperthermia. The injection of chloropromazine alone in rabbits resulted in some significant fall in their body temperature ($P < 0.05$).

The injection of the 5-hydroxytryptamine (5-HT) precusor, 5-HTP in furazolidone pretreated rabbits, resulted in motor restlessness, excitation, tremors and hyperpyrexia which caused the death of 3 out of 4 animals (Figure 2).

There was no protection against the furazolidone-pethidine interaction following pretreatment of the rabbits with α-methyl-*p*-tyrosine (Figure 2) and 2 out of 4 rabbits died in hyperpyrexia 75 min after pethidine injection.

Pretreatment with oxytetracycline or DDT

Pretreatment of rabbits with oxytetracycline resulted in complete protection against the furazolidone-pethidine interaction (Figure 3). On the other hand, pretreatment of rabbits with DDT resulted in acceleration and potentiation of the interaction. Thus there was fatal hyperpyrexia after three days of furazolidone treatment instead of the usual four days period, and 5 out of 6 animals died in hyperpyrexia. Even after two days of furazolidone administration motor restlessness,

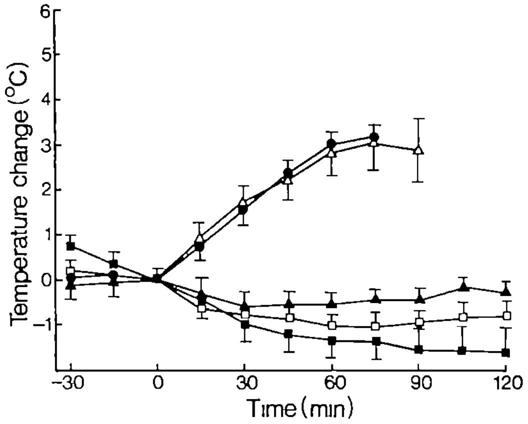

Figure 2 Effects of pretreatment of rabbits with *p*-chlorophenylalanine (PCPA), cyproheptadine, chloropromazine or α-methyl-*p*-tyrosine on the furazolidone-pethidine interaction and interaction of furazolidone with 5-hydroxytryptophan (5-HTP). Pethidine (5 mg/kg i.v.) was injected at time zero in all experimental groups except one (●), where 5-HTP was injected instead. All rabbits were pretreated with furazolidone; in addition the following drugs were administered at the times and doses indicated in the text. PCPA (▲), cyproheptadine (□), chloropromazine (■), α-methyl-*p*-tyrosine (△). Each curve represents the mean response from four rabbits. Vertical lines indicate s.e. mean.

severe tremor and hyperexcitability were precipitated in DDT pretreated rabbits by pethidine injection but there was no significant hyperpyrexia. DDT pretreatment alone followed by pethidine injection resulted in the immediate precipitation of tremor-like convulsions, loss of righting reflex and some non-significant hyperpyrexia (Figure 3). The rabbits recovered completely 60 min later.

Discussion

Furazolidone is used as a local antibacterial agent for the treatment of gastroenteritis in tropical regions. Its MAO inhibiting properties have been described in rats and man by Stern *et al.* (1967) and Pettinger *et al.* (1968). The use of furazolidone for more than five days in patients is not advised since it may subject them to the hazards of hypertensive crises (Pettinger *et al.*, 1968). Although it is not itself a MAO inhibitor, it is believed to be converted to an active metabolite, possibly 2-hydroxyethyl hydrazine, in the rat (Stern *et al.*, 1967). However, Pettinger *et al.* (1968) failed to detect any metabolite which

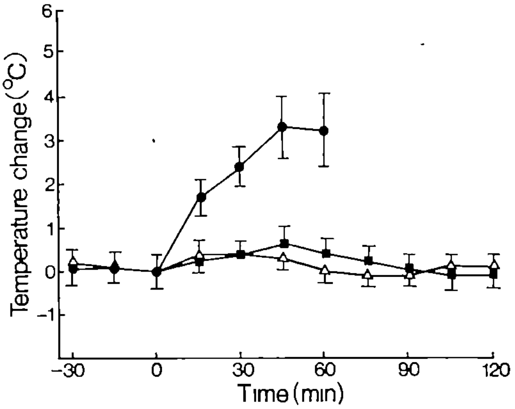

Figure 3 Effects of oxytetracycline or DDT on the furazolidone-pethidine interaction. Pethidine (5 mg/kg i.v.) was injected at time zero in all experiments. (●) Rabbits pretreated with both DDT and furazolidone (six rabbits), (△) rabbits pretreated with both oxytetracycline and furazolidone (five rabbits), (■) rabbits pretreated with DDT alone (four rabbits). Vertical lines indicate s.e. mean.

possessed MAO inhibiting properties in the urine from patients treated with furazolidone.

In the present studies it was possible to demonstrate signs of hyperexcitability, motor restlessness and slight hyperpyrexia in rabbits when pethidine was injected after only two days of furazolidone pretreatment. However, severe interaction and fatal hyperpyrexia resulted when pethidine was injected into rabbits pretreated for four days with furazolidone administered orally. The following results suggest that 5-HT is involved in the interaction occurring between furazolidone and pethidine. Pretreatment with PCPA, which selectively depletes brain 5-HT (Koe & Weissmann, 1966), completely prevented the fatal hyperpyrexic interaction. Also both chloropromazine and cyproheptadine antagonized the furazolidone-pethidine interaction and protected the rabbits. On the other hand the injection of 5-HTP in doses which had little effect on temperature in normal rabbits (Sinclair, 1973) produced severe and fatal hyperpyrexia in furazolidone pretreated rabbits. Fahim *et al.* (1972) have shown that pretreatment with drugs like lithium and yohimbine which increase 5-HT concentration in the brain caused pethidine to provoke a hyperpyrexic response in rabbits. Depletion of brain catecholamines by pretreatment with α-methyl-*p*-tyrosine had no effect on the furazolidone-pethidine interaction.

Pethidine, the tricyclic antidepressants, some antihistamines and dextromethorphan have been shown to block the neuronal uptake of 5-HT

(Carlsson, Corrodi, Fuxe & Hokfelt, 1969; Carlsson, Jonason & Lindqvist, 1969; Carlsson & Lindqvist, 1969; Sinclair, 1973). All of these agents exhibit a characteristic interaction with MAO inhibitors in rabbits (Nymark & Nielsen, 1963; Loveless & Maxwell, 1965; Penn & Rogers, 1971; Fahim *et al.*, 1972; Sinclair, 1972, 1973).

The exact site where conversion of furazolidone into the active metabolite takes place is not known with certainty (Stern *et al.*, 1967). Both the intestinal mucosa and the liver have been suggested as primary sites for biotransformation of furazolidone. Pretreatment of the rabbits with oxytetracycline, which inhibits the gut microflora completely, antagonized the furazolidone-pethidine interaction and prevented the fatal hyperpyrexia. In addition, when furazolidone was injected subcutaneously in a dose of 50 mg/kg daily for four successive days into rabbits, this resulted only in a mild interaction with no hyperpyrexia when pethidine was injected. These findings suggest that the intestinal microflora, rather than the gut wall, is the main site for transformation of furazolidone into the active MAO inhibitor metabolite. Walker (1970) has shown that the liver is not the only site for azo compounds reduction, for the gut microflora also plays an important role in this reaction. It has been shown by Gingel, Bridges & Williams (1971) that pretreatment of rats with antibiotics results in a marked decrease of azo reduction of neoprontozil or prontozil to sulphanilamide.

On the other hand pretreatment with the known drug-metabolizing enzyme inducer, DDT (Hart & Fouts, 1963), resulted in the enhancement, potentiation and acceleration of the furazolidone-pethidine interaction. It is unlikely that DDT enhances transformation of furazolidone by the gut microflora. However, although furazolidone is poorly absorbed from the gut, enough may reach the liver to be more efficiently transformed following treatment with DDT.

The use of furazolidone on long-term basis in diseases such as chronic ulcerative colitis, gastroenteritis and in typhoid carriers has been considered in tropical countries. In the light of the above evidence the use of furazolidone for even a few days may carry the risk of serious interaction with many agents, especially in patients exposed to the pesticide DDT and other persistent halogenated pesticides that induce drug-metabolizing enzymes.

We are grateful to Mr Mahdi El-Helo and to Salah Abdel Rahim for skilful technical assistance. Thanks are also due to Mr Ali El-Tinai for photographic assistance.

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(Received March 24, 1975.
Revised July 4, 1975.)

Br. J. Pharmac. (1975), 55, 503-514

THE DEVELOPMENT OF ANTISERA TO PROSTAGLANDINS B_2 AND $F_{2\alpha}$ AND THEIR ANALYSIS USING SOLID-PHASE AND DOUBLE ANTIBODY RADIOIMMUNOASSAY METHODS

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1 A method for the production of highly substituted prostaglandin-bovine serum albumin conjugates has been developed.

2 Antisera to prostaglandins B_2 and $F_{2\alpha}$ were raised in rabbits immunized with prostaglandin-bovine serum albumin conjugates.

3 The antisera were assessed for specificity and sensitivity by the double antibody radioimmunoassay method and after they were covalently linked to powdered cellulose to form a 'solid-phase' system.

4 Solid phase radioimmunoassays were developed using conventional shaking and in the presence of sucrose which obviates the need for continuous mixing of the incubates.

Introduction

There have been numerous reports on the development of antisera to prostaglandins of the B, F, A and E series, and the use of these antisera for radioimmunoassay of their respective prostaglandins (Caldwell, Burstein, Brock & Speroff, 1971; Jaffe, Smith, Newton & Parker, 1971; Levine, Gutierrez, Cernosek & van Vunakis, 1971, Stylos & Rivetz, 1972; Yu & Burke, 1972; Zusman, Caldwell, Speroff & Behrman, 1972; Jaffe, Behrman & Parker, 1973). Radioimmunoassays have been developed using the double antibody (Levine *et al.*, 1971, Stylos & Rivetz, 1972; Yu & Burke, 1972), dextran coated charcoal (Caldwell *et al.*, 1971; Zusman *et al.*, 1972; Jaffe *et al.*, 1973), and ammonium sulphate precipitation (Jaffe *et al.*, 1971) methods to separate the free from antibody-bound prostaglandin.

We now describe the production of antisera to prostaglandin B_2 and prostaglandin $F_{2\alpha}$ and their analysis by both solid phase and double antibody radioimmunoassay methods. Solid phase radioimmunoassay which is based upon the use of antibodies that have been covalently linked to cellulose is a new technique for prostaglandin estimation.

Antisera were raised in rabbits immunized with prostaglandin-bovine serum albumin conjugates. A method was developed for producing highly substituted conjugates.

Methods

Production of antibodies

Conjugate formation Prostaglandins B_2 and $F_{2\alpha}$ were conjugated to bovine serum albumin (BSA) by the mixed anhydride method described for oestrone (Erlanger, Borek, Beiser & Lieberman, 1959).

Preparation of prostaglandin $F_{2\alpha}$-bovine serum albumin conjugate Prostaglandin $F_{2\alpha}$ (7 mg, 0.02 mM) and $[9-{}^3H_2]$-prostaglandin $F_{2\alpha}$ (49,389 d/min) were transferred in methanol to a 20 ml glass vial. The methanol was evaporated in a stream of N_2 and the residue dried in a vacuum desiccator. Dioxan (0.5 ml), tri-*n*-butylamine (9.5 µl, 0.04 mM, Kodak Ltd.) and isobutylchloroformate (2.1 µl, 0.02 mM, Kodak Ltd.) were then added to the prostaglandin. This solution was cooled to 0° C and stirred by the addition of a small magnetic stirrer (0.5 x 0.1 mm) for 30 minutes. This procedure esterified the mixed anhydride.

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The prostaglandin solution was then added to a 20 ml glass vial containing BSA (23 mg, 0.38 µM, Armour Pharmaceutical Co. Ltd.) dissolved in water (0.6 ml) and 1 N NaOH (23 µl). Aliquots of dioxan (a total of 0.5 ml) were used to rinse out any remaining prostaglandin from the original reaction vessel into the vial containing the solution of BSA. This latter solution was stirred continuously for 4 h and kept cool by surrounding it with ice-cold water. During this period, the prostaglandin $F_{2\alpha}$-BSA conjugation occurred. The solution was then transferred to Visking dialysis tubing (30-32) and dialysed against running tap water for 48 h in order to remove unconjugated prostaglandin. After dialysis, the solution was centrifuged for 20 min at 40° C and 15,000 *g*. The supernatant was freeze-dried to yield the prostaglandin $F_{2\alpha}$-BSA conjugate.

In order to determine the number of moles of prostaglandin $F_{2\alpha}$ bound to 1 molecule of BSA, two small amounts (0.3-0.7 mg) of conjugate were weighed accurately and transferred to separate scintillation vials. The conjugates were digested by warming with redistilled water (0.2 ml) and Nuclear Chicago Scintillant solubilizer (2 ml). Toluene scintillant (10 ml) was added to each solution and they were monitored for radioactivity in a Nuclear Chicago liquid scintillation counter. Once the number of disintegrations per minute (d/min) in a given weight of prostaglandin conjugate was known, the number of moles of prostaglandin $F_{2\alpha}$ bound to one mole of BSA could be calculated since the weight of prostaglandin $F_{2\alpha}$ and the number of d/min of [$^3H_2$]-prostaglandin $F_{2\alpha}$ originally used were known.

In a second system used later, the amounts of BSA, water and 1 N NaOH in the second reaction vessel were halved.

Prostaglandin B_2-BSA conjugates were prepared by identical methods to those used for prostaglandin $F_{2\alpha}$.

Injection regimen

Twelve adult male New Zealand white rabbits (approximately 2 kg) were used. Six were injected with prostaglandin $F_{2\alpha}$ conjugate and six with B_2 conjugate. The back of each animal was shaved and under sterile conditions it was injected intradermally, at approximately 50 sites, with a total of 2 ml of an emulsion containing prostaglandin conjugate (100 µg) in Freund's complete adjuvant. Pertussis vaccine (0.5 ml) was then injected subcutaneously to prime the animal's immune responses (Vaitukaitis, Robbins, Nieschlag & Ross, 1971). Boost injections of conjugate, emulsified in Freund's incomplete adjuvant, were administered subcutaneously at four sites (suprascapular and femoral) 26 weeks later and subsequently at 6-weekly intervals. The first and second boost injections contained 100 µg of prostaglandin conjugate and later injections contained 1 mg of conjugate.

Blood collection

Rabbits were bled at 4-weekly intervals until the first boost and thereafter at 6-weekly intervals, one week after each subsequent boost. During blood collections, the rabbit was restrained in a box, xylene was applied to one ear to increase the blood flow, soft yellow paraffin wax was applied to both surfaces of the ear in the region to be incised and, under sterile conditions, a stab incision was made in the marginal vein. Blood dripped from the vein over the surface of the soft paraffin wax and into a large glass centrifuge tube. At each bleed 50 ml of blood was collected from every rabbit.

The blood was allowed to clot at room temperature then left at 4° C overnight. The serum (containing some cells) was collected and kept at 4° C. The clot was warmed to room temperature and additional serum was removed. The serum was centrifuged at 1000 *g* for 15 to 20 min to remove a small cellular deposit, then decomplemented by incubation at 56° C for 30 min and finally cooled to room temperature. The volume of serum was measured and sodium azide (0.1 ml of a 10 mg/ml solution per 10 ml of serum) was added to it. It was then dispensed in 2 ml volumes in sterile McCartney bottles (5 ml) and stored at −20° C until use.

Reagents for radioimmunoassay

Double antibody radioimmunoassay method A diluent with the following composition was used; 0.05 M tris-hydrochloride buffer (pH 8), sodium azide (0.1 g/l) and gelatine (1 g/l). Tritiated prostaglandin $F_{2\alpha}$ (35.6 ng/µCi; New England Nuclear Company), [$^3H_2$]-prostaglandin $F_{2\alpha}$ (2.2 ng/µCi) and [$^3H_2$]-prostaglandin B_2 (2.5 ng/µCi; Amersham) were dissolved in methanol and stored in siliconized vials at −20° C. Before use, the methanol was evaporated in a stream of N_2 and the residue dissolved in diluent to give the following concentrations: 3 ng/ml of 35.6 ng/μCi [$^3H_2$]-prostaglandin $F_{2\alpha}$ (i.e. 150 pg/50 μl), 0.2 or 0.6 ng/ml of 2.2 ng/μCi [$^3H_2$]-prostaglandin $F_{2\alpha}$ (i.e. 10 or 30 pg, respectively/50 μl) and 0.2 or 0.4 ng/ml of 2.5 ng/μCi [$^3H_2$]-prostaglandin B_2 (i.e. 10 or 20 pg, respectively/50 μl). These are referred to below as the tracer solutions.

Donkey anti-rabbit serum (Wellcome Reagents Ltd.) was stored at −20° C in 25 ml aliquots until use. After thawing it was kept at 4° C for periods of approximately 4 weeks during use. A stock solution was prepared by diluting 3 ml of serum to 10 ml with diluent. Normal rabbit serum was obtained from non-immunized donors by the techniques already described and a stock solution was prepared by diluting the serum 1 in 53 with diluent. The methods for determining the concentrations of normal rabbit serum and donkey anti-rabbit serum required for a radioimmunoassay have been described by Hunter (1973).

A scintillant with the following composition was used for both the double antibody and solid phase radioimmunoassay methods: naphthalene, 75 g; PPO (2,5, diphenyloxazole), 7 g; DMPOPOP (1,4-di-(2-(4 methyl-5-phenyloxazolyl)-benzene), 0.6 g; 2-ethoxyethanol 600 ml, toluene 1 litre. A volume of 13 ml of this scintillant absorbs 0.6 ml of an aqueous solution. Counting efficiency is then approximately 30%.

Solid phase radioimmunoassay method Two diluents were employed in the solid phase radioimmunoassay method. The first, a diluent identical to that described for the double antibody method (referred to subsequently as diluent), and the second, a sucrose diluent (always referred to as sucrose diluent). The sucrose diluent was composed of 0.05 M tris-hydrochloride buffer (pH 8), sodium azide (0.1 g/l), gelatine (1 g/l) and sucrose 200 g/l). Tritiated prostaglandin $F_{2\alpha}$ (2.2 ng/μCi) and [$^3H_2$]-prostaglandin B_2 (2.5 ng/μCi) were used. Solutions in diluent of 0.6 ng/ml of [$^3H_2$]-prostaglandin $F_{2\alpha}$ (30 pg/50 μl) and 0.4 ng/ml of [$^3H_2$]-prostaglandin B_2 (20 pg/50 μl) were made for radioimmunoassay.

An antiserum-cellulose complex as used by Wide (1969) and prepared according to the method of Bolton, Dighe & Hunter (1974), was used for the solid phase radioimmunoassays. A 1 in 100 dilution of the antiserum in the antiserum-cellulose complex was prepared as a stock solution and stored at 4°C. This dilution was made using diluent with a sodium azide concentration of 20 g/litre.

Dilution curves

Double antibody radioimmunoassay method The serum (0.1 ml) from each injected rabbit was diluted with diluent to give a 1 in 100 stock solution. This solution was used to prepare a series of 2-fold dilutions (1 ml each), ranging from 1 in 1000 up to 1 in 256,000. Tracer solution (50 μl) was then added to each dilution and the solutions were mixed and incubated for 1 h at room temperature (18-20° C). After incubation, normal rabbit serum (50 μl of a 1 in 53 dilution) was added to each solution and after mixing and standing for 10 min to equilibrate, donkey anti-rabbit serum (50 μl of a 3 in 10 dilution) was added to all tubes. (At a serum dilution of 1 in 1270, the rabbit gamma globulin present required a 1 in 80 dilution of the donkey anti-rabbit serum for adequate precipitation). The contents of the tubes were mixed and incubated for 16 h at 4° C. After incubation, the samples were centrifuged at 4° C, 1200 *g* for 45 min, then 0.6 ml of the supernatant was removed from each tube and added to a vial containing 13 ml of scintillant. The vials were monitored for radioactivity in a Nuclear Chicago liquid scintillation counter, using the external standards channels/ratio method and counting each vial for 4 or 10 minutes. Each dilution curve also included 4 blanks (non-specific binding control tubes) and 4 counting standards. The blanks contained 1 ml of diluent, 50 μl of tracer solution, 50 μl of diluted normal rabbit serum and 50 μl of diluted donkey anti-rabbit serum. The counting standards contained 1 ml of diluent and 50 μl of tracer solution. Counts from the liquid scintillation counter were recorded on a paper tape which was fed into a PDP8 computer programmed to calculate disintegrations per minute (d/min), counting efficiency and percentage binding of the tracer by the antiserum. The latter parameter was calculated by the following equation:

$$\text{Percentage bound} = 100 - \frac{(\text{d/min} \times 100)}{\text{average counting standard d/min}}$$

If the non-specific binding, as shown by the blanks, exceeded 10% in any experiment, the experiment was repeated.

A dilution curve (percentage binding of the tracer by the antiserum against dilution of antiserum) was drawn for each antiserum and the 'titre' was read as that dilution which bound 60% of the tracer in the absence of non-radioactive standard prostaglandin.

Solid phase radioimmunoassay method The solid phase shaking method was employed for dilution curves. A series of 2-fold dilutions in 1 ml of diluent, ranging from 1 in 1000 up to 1 in 512,000 was prepared in duplicate from the stock solution (1 in 100) of the prostaglandin antiserum-cellulose complex. This stock solution was stirred continuously, with a magnetic stirrer, while aliquots were removed from it for the preparation of antiserum dilutions. Tracer solution (50 μl) was added to each dilution of antiserum. These solutions were mixed then shaken without stoppering on a horizontal agitator for 2 h at room temperature (18-20° C). After incubation the samples were centrifuged at 4° C, 1200 *g* for 15 min then 0.6 ml of the supernatant was removed from each tube and added to a vial containing 13 ml of scintillant. The vials were monitored for radioactivity (using 4 min counts) and the percentage binding of the tracer by the antiserum was calculated as in the double antibody dilution curve method. A standard curve was plotted for each antiserum and the 'titre' was determined.

Each dilution curve also included 4 counting standards which contained 1 ml of diluent and 50 μl of tracer solution.

Standard curves

Double antibody radioimmunoassay method Standard curves were obtained only for antisera with high 'titres'. A dilution of the antiserum was prepared so that when 50 μl was added to the radioimmunoassay tube (10 x 75 mm) in which the antibody reaction took place (a total volume of 1.2 ml) the final dilution was equal to the 'titre'.

Tubes containing standard prostaglandin solutions (1 ml) were set up in duplicate (Table 1). In addition, zero standards, blanks and counting standards were set up in quadruplicate, each containing 1 ml of diluent. Tracer solution (50 μl) was added to each tube and the solutions were mixed and equilibrated for 10 minutes. Diluted antiserum (50 μl) was then added to every tube, except the blanks and counting standards, and after mixing, the samples were incubated at room temperature (18-20° C) for 1 hour. After incubation, the procedure for a standard curve was identical to that performed for a dilution curve.

A standard curve (percentage binding of the tracer by the antiserum against weight of prostaglandin) was drawn for each antiserum. The detection limit of each radioimmunoassay was defined loosely and for comparative purposes only as the amount of standard prostaglandin required to give a 10% fall in binding from the zero standard under these closely defined conditions. Binding of approximately 60% was obtained with the zero standards.

Solid phase radioimmunoassay method Standard curves were obtained for prostaglandin $F_{2\alpha}$ antiserum (rabbit number 6, 3rd boost) and for prostaglandin B_2 antiserum (rabbit number 7, 1st boost). Both the shaking and the sucrose methods for solid phase radioimmunoassay were performed for prostaglandin $F_{2\alpha}$ antiserum but the shaking method only was performed for prostaglandin B_2 antiserum. A dilution of the stock solution of the prostaglandin antiserum-cellulose complex was prepared so that when 0.5 ml was added to each radioimmunoassay tube (15 x 75 mm) in which the antibody reaction took place (a total volume

Table 1 Amounts (ng) of prostaglandin B_2 (PGB_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) used as standards for double antibody and solid phase radioimmunoassay

<table>
<tr><th colspan="3">Double antibody standards</th><th colspan="2">Solid phase standards</th></tr>
<tr><th>$PGF_{2\alpha}$ (35.6 ng/μCi)</th><th>$PGF_{2\alpha}$ (2.2 ng/μCi)</th><th>PGB_2 (2.5 ng/μCi)</th><th>$PGF_{2\alpha}$ (2.2 ng/μCi)</th><th>PGB_2 (2.5 ng/μCi)</th></tr>
<tr><td>0.15</td><td colspan="2">0.01</td><td colspan="2">0.01</td></tr>
<tr><td>0.45</td><td colspan="2">0.02</td><td colspan="2">0.02</td></tr>
<tr><td>1.05</td><td colspan="2">0.03</td><td colspan="2">0.03</td></tr>
<tr><td>2.25</td><td colspan="2">0.07</td><td colspan="2">0.05</td></tr>
<tr><td>4.65</td><td colspan="2">0.15</td><td colspan="2">0 07</td></tr>
<tr><td>9.45</td><td colspan="2">0.31</td><td colspan="2">0.1</td></tr>
<tr><td>19.05</td><td colspan="2">0.63</td><td colspan="2">0.15</td></tr>
<tr><td></td><td colspan="2">1.27</td><td colspan="2">0.3</td></tr>
<tr><td></td><td colspan="2">2.55</td><td colspan="2">0.6</td></tr>
<tr><td></td><td colspan="2"></td><td colspan="2">1.2</td></tr>
</table>

In the double antibody method these amounts were added to each radioimmunoassay tube in 1 ml of diluent. In the solid phase method double these amounts were added to each assay tube in 0.5 ml of diluent and they were diluted by the addition of the antiserum-cellulose complex in 0.5 ml of diluent. The specific activity of the tracer used is in brackets.

of 1.05 ml), the final dilution was equal to the 'titre'.

Tubes containing standard prostaglandin solutions (0.5 ml) were set up in duplicate (Table 1). In addition, zero standards and blanks (sucrose method only) containing 0.5 ml of diluent, and counting standards containing 1 ml of diluent, were set up in quadruplicate. Tracer solution (50 μl) was added to each tube and the solutions were mixed and left to equilibrate for 10 min at room temperature (18-20° C). The procedure for the shaking and the sucrose methods now differed slightly.

Shaking method Diluted antiserum, 0.5 ml in diluent, was added to every tube except the counting standards and, after mixing, the samples were shaken at room temperature (18-20° C) for 2 hours. (No blanks were included in this method as they would have been identical to the counting standards.

Sucrose method Diluted antiserum, 0.5 ml in sucrose diluent, was added to every tube, except the blanks and counting standards. Sucrose diluent (0.5 ml) was added to the blanks. After mixing, the samples were left to incubate without shaking at room temperature (18-20° C) for 3 hours.

After incubation using the shaking or the sucrose methods, all the samples were centrifuged and sampled according to the methods described for the solid phase dilution curves.

Standard curves were plotted, as in the double antibody radioimmunoassay method, and the sensitivity of each assay was determined.

Optimum time for incubation of antibody with antigen

Double antibody radioimmunoassay method Four sets of two zero standards were prepared as for a standard curve. Incubation periods of 1, 2, 4 and 24 h were then left before adding the diluted normal rabbit serum and the diluted donkey anti-rabbit serum.

Solid phase radioimmunoassay method Four sets of two zero standards were prepared as for a standard curve, using the sucrose (prostaglandin $F_{2\alpha}$ only) and shaking (prostaglandin $F_{2\alpha}$ and B_2) methods. The samples were incubated for periods of 0.5, 1, 2, 3 and 4 h before centrifuging.

Effect of varying tracer concentrations

Double antibody radioimmunoassay method A standard curve was set up in which 10 pg of tracer was added to all the samples and a tracer curve in which four sets of two tubes containing 20, 40, 80 and 160 pg of tracer in a total of 1 ml of diluent was prepared. The procedure for adding antiserum, normal rabbit serum and donkey anti-rabbit serum was then followed, as for a standard curve.

Graphs of percentage binding of the tracer by the antiserum against weight (pg) of prostaglandin were then plotted for the standard and the tracer samples. Since 10 pg of tracer was added to all the samples in the standard curve, the tracer samples, (containing a total of 20, 40, 80 and 160 pg) were equivalent to 10 + 10, 10 + 30, 10 + 70 and 10 + 150 pg of tracer prostaglandin. Thus, in order to compare the tracer samples with the standards, a graph was plotted in which the percentage of tracer bound was plotted against tracer prostaglandin amounts of 10, 30, 70 and 150 pg (see Figure 4).

Solid phase radioimmunoassay method Standard and tracer curves for prostaglandins B_2 and $F_{2\alpha}$ were prepared using the same principles as for the double antibody radioimmunoassay method. The standard curves were obtained using 20 pg of tracer prostaglandin B_2 and 30 pg of tracer prostaglandin $F_{2\alpha}$. The shaking and sucrose solid phase radioimmunoassay methods were used for prostaglandin $F_{2\alpha}$ and the shaking method only was used for prostaglandin B_2.

Specificity studies

Double antibody and solid phase methods A standard curve for the prostaglandin to which the antiserum was raised was set up. In parallel, standard curves for different prostaglandins were prepared, starting with a prostaglandin concentration of 0.1 ng/ml and making four-fold dilutions. All dilutions were set up in duplicate.

The percentage cross reactivity of the prostaglandin B_2 or $F_{2\alpha}$ antisera with another prostaglandin (e.g. prostaglandin A_2) was determined by finding the concentration of prostaglandin A_2 which corresponded with a 10% fall from the binding obtained with the zero standard. The following calculation was then applied:

e.g. percentage cross reactivity of prostaglandin B_2 (PGB_2) antiserum with prostaglandin A_2 (PGA_2) =

$$\frac{\text{Concentration of } PGB_2 \text{ at 10\% fall from binding of zero standard}}{\text{Concentration of } PGA_2 \text{ at 10\% fall from binding of zero standard}} \times 100$$

Results

Antisera to prostaglandins B_2 and $F_{2\alpha}$ were raised in rabbits immunized with prostaglandin-bovine serum albumin conjugates. The 'titres' of all the antisera were determined using the double antibody radioimmunoassay method, and standard curves, optimum incubation times, tracer studies and specificity studies were performed for antisera with high 'titres'.

As a result of these studies prostaglandin $F_{2\alpha}$ antiserum from rabbit number 6 (3rd boost) and prostaglandin B_2 antiserum from rabbit number 7 (1st boost) (Table 2) were selected for the routine estimation of prostaglandins $F_{2\alpha}$ and B_2, by the double antibody radioimmunoassay method and for the development of a solid phase radioimmunoassay method.

Solid phase radioimmunoassay was performed using both the shaking and sucrose methods. The shaking method was developed for both prostaglandin B_2 and $F_{2\alpha}$ antisera but the sucrose method has, as yet, only been developed for the prostaglandin $F_{2\alpha}$ antiserum. Standard curves, optimum incubation times, tracer and specificity studies were performed for the prostaglandin B_2 and $F_{2\alpha}$ antisera using the solid phase radioimmunoassay methods.

Conjugate formation

The first conjugation procedure yielded conjugates with between 20-37 moles of prostaglandin $F_{2\alpha}$ and 18-26 moles of B_2 bound to one mole of BSA.

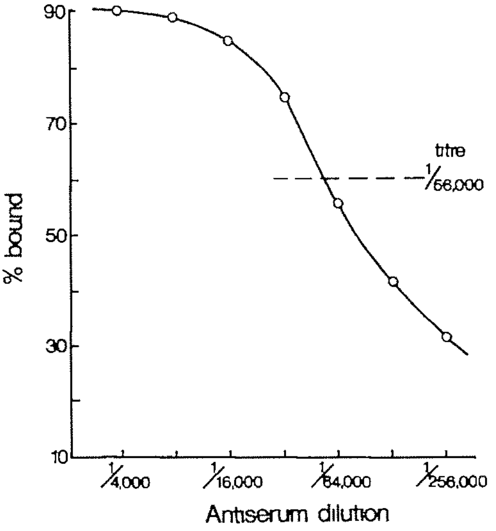

Figure 1 Dilution curve for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) antiserum, obtained from rabbit number 6 (3rd boost). Double antibody radioimmunoassay method used with 10 pg of $[^3H_2]$-prostaglandin $F_{2\alpha}$ (2.2 ng/μCi).

The second system resulted in 50 moles of prostaglandin $F_{2\alpha}$ and 36 moles of prostaglandin B_2 being bound to one mole of BSA. These very highly substituted conjugates were particularly effective in increasing the antibody 'titre' in injected rabbits.

Testing of antisera

Dilution curves for double antibody radioimmunoassay method These were obtained for all the antisera and their 'titres' were determined. In

Table 2 The highest antiserum 'titres' produced by each rabbit

Prostaglandin	*Rabbit number*	*'Titre'*	*Boost number*
$F_{2\alpha}$	1	1 in 51,200	5
	2	Died	—
	3	1 in 70,400	4
	4	1 in 108,800	4,5.
	5	1 in 20,800	5
	6	1 in 76,800	5
B_2	7	1 in 35,200	5
	8	1 in 17,600	4,5.
	9	1 in 13,600	3
	10	Died	—
	11	1 in 54,400	6
	12	Died	—

The 'titres' were obtained from dilution curves performed using the double antibody radioimmunoassay method and 20 pg of $[^3H_2]$-prostaglandin B_2 (2.5 ng/μCi) or 10 pg of $[^3H_2]$-prostaglandin $F_{2\alpha}$ (2.2 ng/μCi). Rabbits 1-6 were injected with prostaglandin $F_{2\alpha}$ conjugate and rabbits 7-12 were injected with B_2 conjugate.

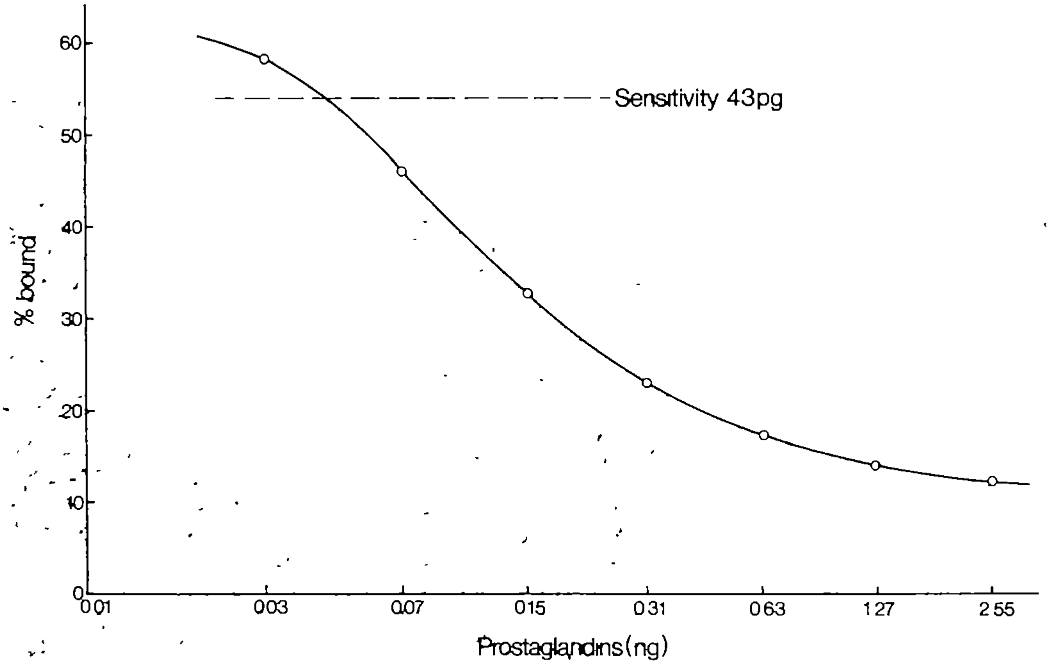

Figure 2 Standard curve for prostaglandin B$_2$ (PGB$_2$) using the double antibody radioimmunoassay method. Antiserum obtained from rabbit number 7 (1st boost); 20 pg of [^{3}H$_2$]-prostaglandin B$_2$ (2.5 ng/µCi) used; 'titre' of antiserum 1 in 24,800. An average binding of 64% was obtained from the 4 zero standards.

rabbits injected with prostaglandins B$_2$ and F$_{2\alpha}$ conjugates, antisera with 'titres' as high as 1 in 54,400 (20 pg tracer) and 1 in 108,800 (10 pg tracer), respectively, were obtained (Table 2).

A dilution curve for prostaglandin F$_{2\alpha}$ antiserum, obtained from rabbit number 6 (3rd boost), is illustrated in Figure 1.

Dilution curves for solid phase radioimmunoassay method These were obtained for prostaglandin B$_2$ antiserum from rabbit number 7 (1st boost) and for prostaglandin F$_{2\alpha}$ antiserum from rabbit number 6 (3rd boost). The shaking method for solid phase radioimmunoassay was used.

The 'titres' of these antisera (as determined by double antibody radioimmunoassay) were reduced using the solid phase radioimmunoassay method (Table 3).

Standard curves for double antibody and solid phase radioimmunoassay methods Standard curves were prepared (using the double antibody radioimmunoassay method) for all antisera with high 'titres'. Standard curves for prostaglandins B$_2$ and F$_{2\alpha}$ are illustrated in Figures 2 and 3. The detection limit of each radioimmunoassay was determined from the standard curve and these limits for prostaglandins B$_2$ and F$_{2\alpha}$, using the

Table 3 The 'titres' of two antisera, as determined by solid phase and double antibody radioimmunoassay (RIA) methods

			'Titre'	
Prostaglandin	*Antiserum used*	*Tracer used*	*Double antibody RIA*	*Solid phase RIA (Shaking Method)*
F$_{2\alpha}$	Rabbit number 6 (3rd boost)	30 pg (2.2 ng/µCi)	1 in 38,400	1 in 8,800
B$_2$	Rabbit number 7 (1st boost)	20 pg (2.5 ng/µCi)	1 in 24,800	1 in 9,600

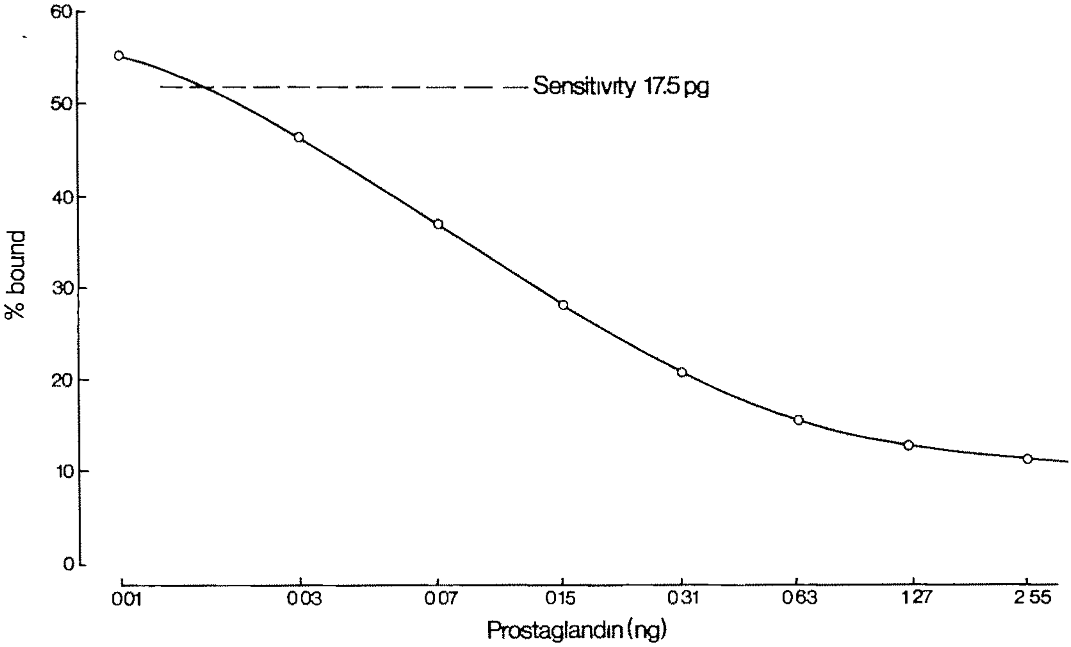

Figure 3 Standard curve for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) using the double antibody radioimmunoassay method. Antiserum obtained from rabbit number 6 (3rd boost); 10 pg of [$^3H_2$]-prostaglandin $F_{2\alpha}$ (2.2 ng/μCi) used; 'titre' of antiserum 1 in 64,000. An average binding of 61.5% was obtained from the 4 zero standards.

double antibody and solid phase radioimmunoassay methods, are illustrated in Table 4. There was little change in the detection limit of the radioimmunoassay for prostaglandin B_2, whether the double antibody or solid phase methods were used, but the detection limit of the assay for prostaglandin $F_{2\alpha}$ decreased when the solid phase (sucrose and shaking) methods were used (Table 4).

Optimum time for incubation of antibody with antigen

The effect of changing incubation time on the efficiency of the antibody-antigen reaction, is illustrated in Table 5. As a result of these experiments, a one hour incubation period was chosen for all radioimmunoassays for prostaglandins B_2 and $F_{2\alpha}$ using the double antibody

Table 4 The effect of the double antibody and solid phase radioimmunoassay methods on the sensitivities of assays for prostaglandin B_2 (PGB_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)

			Sensitivity (pg) of each radioimmunoassay		
				Solid phase method	
PG	*Antiserum used*	*Tracer used (Specific activity)*	*Double antibody method*	*Shaking*	*Sucrose*
B_2	Rabbit number 7 (1st boost)	20 pg (2.5 ng/μCi)	43 s.e. = ± 1.25 $n = 8$	43 s.e. = ± 2.78 $n = 13$	
$F_{2\alpha}$	Rabbit number 6 (3rd boost)	10 pg (2.2 ng/μCi)	17.5		
		150 pg (35.6 ng/μCi)	100 s.e. = ± 6.18 $n = 5$		
		30 pg (2.2 ng/μCi)	27 s.e. = ± 1.54 $n = 5$	51 s.e. = ± 6.22 $n = 7$	60 s.e. = ± 3.68 $n = 13$

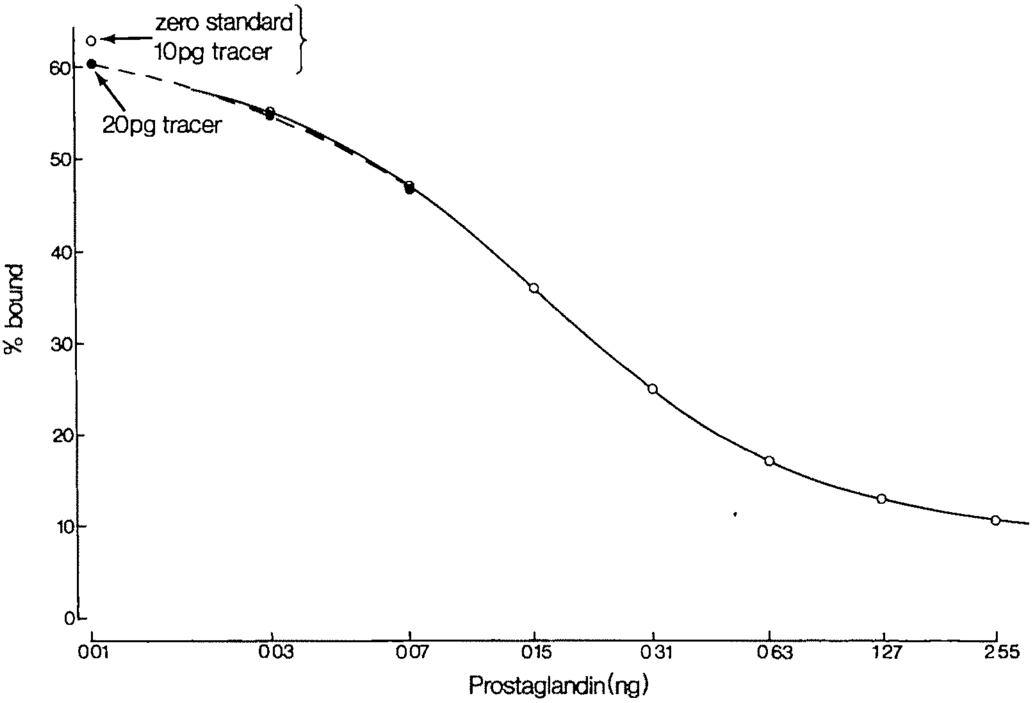

Figure 4 Comparison of standard (o—o) and tracer (●—●) curves for prostaglandin B_2 (PGB_2) using the double antibody radioimmunoassay method. Antiserum obtained from rabbit number 7 (1st boost), 10 pg of [$^3H_2$]-prostaglandin B_2 (2.5 ng/μCi) used in standard curve.

Table 5 Effect of changing incubation time (h) on the development of the antibody-antigen reaction

RIA Method	*PG*	*Antiserum from rabbit*	*Tracer (Specific activity)*	*Incubation time (h)*					
				0.5	*1*	*2*	*3*	*4*	*24*
Double antibody	$F_{2\alpha}$	Number 6 (3rd boost)	150 pg (35.6 ng/μCi)		67.6	69.1		69.6	68.8
	$F_{2\alpha}$	Number 6 (3rd boost)	10 pg (2.2 ng/μCi)		63.7	65.8		66.8	65.8
	B_2	Number 7 (1st boost)	20 pg (2.5 ng/μCi)		64.3	66.1		66.9	65.9
Solid phase									
Shaking method	$F_{2\alpha}$	Number 6 (3rd boost)	30 pg (2.2 ng/μCi)	47.4	55.0	58.5	57.8		
Sucrose method	$F_{2\alpha}$	Number 6 (3rd boost)	30 pg (2.2 ng/μCi)		56.5	57.0	60.1	60.1	
Shaking method	B_2	Number 7 (1st boost)	20 pg (2.5 ng/μCi)	59.1	62.5	66.0	64.1		

The results, which are the average of duplicate zero standards, show the percentage of tracer prostaglandin bound to antiserum after various incubation times.

method. When the solid phase radioimmunoassay methods were employed for prostaglandins B_2 and $F_{2\alpha}$ estimation, incubation periods of 2 and 3 h were used for the shaking and sucrose methods, respectively.

Effect of varying tracer concentrations

Double antibody radioimmunoassay method The effect of varying tracer concentrations of prostaglandin B_2 is shown in Figure 4. Since the standard and tracer curves are almost superimposable the mass of prostaglandin in the tracer and cold prostaglandin samples is almost identical and as expected the antiserum is unable to distinguish between labelled and unlabelled prostaglandin B_2.

In Figure 4, it can be seen that the difference in binding between using 10 and 20 pg of tracer prostaglandin B_2 was negligible. In all further studies, 20 pg of tracer prostaglandin B_2 was therefore used as it allowed a shorter counting time. After similar experiments with tracer prostaglandin $F_{2\alpha}$, 2.2 and 35.6 ng/μCi, 10 and 150 pg, respectively of the two tracer preparations were used for the double antibody radioimmunoassay method.

Solid phase radioimmunoassay method As a result of varying tracer concentrations using the solid phase radioimmunoassay methods, 20 pg of tracer prostaglandin B_2 (2.5 ng/μCi) and 30 pg of tracer prostaglandin $F_{2\alpha}$ (2.2 ng/μCi) were used routinely.

Specificity studies

The percentage cross reactivity of prostaglandin $F_{2\alpha}$ antiserum from rabbit number 6 (3rd boost) and prostaglandin B_2 antiserum from rabbit number 7 (1st boost) with various prostaglandins is shown in Table 6. Apart from 100% cross-reaction with prostaglandin $F_{1\alpha}$, the prostaglandin $F_{2\alpha}$ antiserum was fairly specific. The 40% cross reactivity with prostaglandin D_2 is significant. The prostaglandin B_2 antiserum exhibited 16-18% cross reaction with prostaglandin B_1 but otherwise it was reasonably specific (Table 6). There was little difference in the specificity of the prostaglandin $F_{2\alpha}$ antiserum whether used by the solid phase or double antibody radioimmunoassay method, but the specificity of the prostaglandin B_2 antiserum appeared to improve when it was used in the solid phase assay method (Table 6)

Some of the specificity studies (double antibody method) for prostaglandin B_2 antiserum from rabbit number 7 (1st boost) are illustrated in Figure 5.

Discussion

Antisera to prostaglandins B_2 and $F_{2\alpha}$ were raised

Table 6 Percentage cross reactivity of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) antiserum from rabbit number 6 (3rd boost) and prostaglandin B_2 (PGB_2) antiserum from rabbit number 7 (1st boost), with other prostaglandins. The mass and specific activity of the tracer prostaglandin used and the 'titre' of the antisera are tabulated

	Double antibody radioimmunoassay method				*Solid phase radioimmunoassay method*	
					Sucrose	*Shaking*
PG tested	*Tracer PG*				*Tracer PG*	
	$F_{2\alpha}$			*B_2*	*$F_{2\alpha}$*	*B_2*
	10 pg	*30 pg*	*150 pg*	*20 pg*	*30 pg*	*20 pg*
	(2.2 ng/μCi)		*(35.6 ng/μCi)*	*(2.5 ng/μCi)*	*(2.2 ng/μCi)*	*(2.5 ng/μCi)*
	Titre	*Titre*	*Titre*	*Titre*	*Titre*	*Titre*
	1 in 64,000	*1 in 38,000*	*1 in 11,200*	*1 in 24,800*	*1 in 8,800*	*1 in 9,600*
E_2	2.5	1.21	0.7	7.0	1.5	0.6
E_1	0.4	0.52	0.5	2 0	3.3	1.3
A_2	0.1	<0 056	0.003	10 0	<0.04	4.7
A_1				6 0		1 7
E_2				9.0		
B_2	0.0017	0.0016	0.1		0 008	
B_1				18.0		15.9
$F_{2\alpha}$				0.004		0.018
$F_{2\beta}$	0.3	0.189	0.3		0.4	
$F_{1\alpha}$	120.0	100 0	73.0		92.1	
15 oxo $F_{2\alpha}$	0.2	2.6	0.2		3.25	
dihydro B_1				2.0		4.4
D_2					40.0	

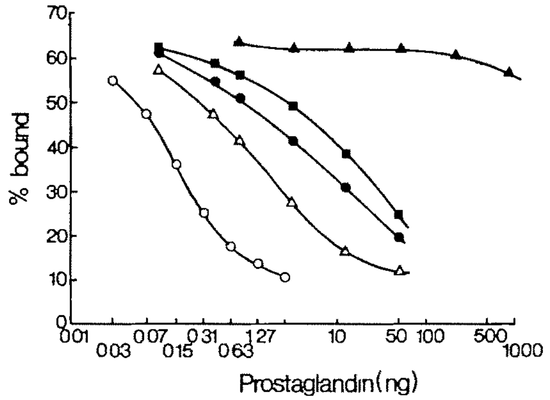

Figure 5 Specificity studies for prostaglandin B_2 (PGB_2) antiserum, obtained from rabbit number 7 (1st boost). The double antibody radioimmunoassay method was used with 20 pg of [$^3H_2$]-prostaglandin B_2 (2.5 ng/μCi), antiserum 'titre' 1 in 24,800. Binding of 63% was obtained with the zero standards (an average of 4 readings). (▲) $F_{2\alpha}$; (■) Dihydro B_1; (●) A_1; (△) B_1, (○) B_2.

in rabbits immunized with prostaglandin-bovine serum albumin conjugates. The antisera were analysed by both the solid phase and double antibody radioimmunoassay methods.

Solid phase radioimmunoassay, in which the prostaglandin antiserum is complexed to cellulose, is a new immunological technique for prostaglandin estimation. In this paper, two solid phase methods (shaking and sucrose) are discussed. Practically, the sucrose method is the simpler of the two since it involves the use of a sucrose diluent which acts as a support medium for the cellulose bound antibody. The contents of the assay tubes therefore do not require shaking.

Solid phase radioimmunoassay has proved to be a highly sensitive and reproducible method for prostaglandin estimation. It has several advantages over the double antibody (Levine *et al.*, 1971; Stylos & Rivetz, 1972; Yu & Burke, 1972), dextran-coated charcoal (Caldwell *et al.*, 1971, Zusman *et al.*, 1972; Jaffe *et al.*, 1973) and ammonium sulphate precipitation (Jaffe *et al.*, 1971) methods which have previously been used for separating free prostaglandin from that bound to antiserum. Its main advantage is that it requires the addition of only two solutions (antiserum and tracer) to each sample, and is, therefore, extremely quick and suitable for handling large numbers of samples. After the addition of antiserum and tracer, only a short incubation period is required before the samples are centrifuged and the supernatant monitored for radioactivity. These procedures contrast sharply with the double antibody method which requires the addition of four solutions to each sample and two incubation periods, one of which lasts overnight. The solid phase method is manipulatively simple and suffers neither from a tendency to high non-specific binding, which is the case with ammonium sulphate, nor from the need for careful timing, as is the case with charcoal.

The solid phase and double antibody methods described in this paper were capable of detecting concentrations as low as 10 pg/ml of prostaglandins B_2 and $F_{2\alpha}$. Apart from the high cross reactivity of the prostaglandin $F_{2\alpha}$ antiserum with $F_{1\alpha}$, and of prostaglandin B_2 antiserum with B_1, the particular antisera used in these assays had low cross reactivities with other prostaglandins tested. The high cross reactivity with prostaglandin D_2 may be a serious limitation in situations where this prostaglandin occurs naturally.

We consider that the solid phase radioimmunoassay method described here has many advantages for workers in the prostaglandin field who wish to measure small quantities of prostaglandins F_α and B in larger numbers of samples. The problems encountered in applying these methods to biological samples and to the estimation of prostaglandins of the E and A series are the subject of a subsequent paper.

We are very grateful to Dr W. Hunter without whose advice and encouragement this work could not have been done. This research was supported by an M.R.C. Programme Grant to Professor E.W. Horton.

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(Received April 25, 1975)

Br. J. Pharmac. (1975), 55, 515-518

PROSTAGLANDINS IN PYOMETRIAL FLUID FROM THE COW, BITCH AND FERRET

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1 Pyometra is a disorder of the uterus usually associated with bacterial infection plus obstruction.

2 Large quantities of fluid often collect in the uterus during this condition.

3 Pyometrial fluid obtained from three species was found to contain prostaglandin $F_{2\alpha}$, usually in large quantities.

4 Prostaglandin E_2 was present in smaller quantities in five of the six samples

5 These findings are discussed in relation to the known occurrence of prostaglandins in inflammatory fluid, and to the problem of infertility.

Introduction

Pyometra is characterized by a progressive collection of pus in the cavity of the uterus and the persistence of functional luteal tissue in the ovary. It is a pathological and inflammatory condition usually associated with uterine infection and infertility. The pus may be watery in nature and present in large quantities. In the majority of cases the cervix is closed, but animals have been seen in which there was a purulent discharge (Arthur, 1964). In the bitch, the so-called 'closed' condition, where fluid accumulates in the uterus, is accompanied by cystic hyperplasia of the endometrium, vaginal discharge, vulval oedema and uterine infection (Dow, 1957).

Inflammatory fluid from several animals obtained from various sites has been found to contain prostaglandins. They are usually prostaglandins of the E series and, where identification has been taken further, prostaglandin E_2 mainly is present (Willis, 1970; Greaves, Søndergaard & McDonald-Gibson, 1971; Ånggård & Jonsson, 1971; Eakins, Whitelocke, Perkins, Bennett & Unger, 1972; Greenwood & Kerry, 1975). Uterine tissue is known to produce and release prostaglandins in several species and prostaglandin $F_{2\alpha}$ is the main prostaglandin formed (see Poyser, 1973, 1974a,b for references). The endotoxin from *Salmonella* enteritis has been found to increase the endometrial levels of prostaglandin fourfold when injected into pregnant mice on day 16. Furthermore, abortion occurred following such treatment. Previous treatment with indomethacin prevented the abortifacient action of the endotoxin, though it did not prevent abortion due to administered prostaglandin $F_{2\alpha}$ (Skarnes & Harper, 1972). The study indicated that prostaglandin released from the uterus in response to bacterial infection could adversely affect fertility in animals.

In the present study, pyometrial fluid has been obtained from the cow, bitch and ferret uterus to study the nature and concentrations of prostaglandins present.

Methods

Pyometrial fluid was obtained during clinical investigations of infertility in three cows, two bitches and one ferret. The fluid was recovered by aspiration from the uterus at slaughter or laparotomy. It was stored at −20° C before assay. After thawing, the pH of the fluid was recorded and lowered to pH 4.5 by the addition of 1 N or 0.1 N HCl. Prostaglandins were extracted by solvents, purified by silicic acid column chromatography and assayed on the rat fundal strip by methods previously described (Blatchley, Donovan, Horton & Poyser, 1972). Following assay, the prostaglandin E and F extracts obtained from the bitch pyometrial fluid were further purified by thin-layer chromatography using the AI solvent system of Grèen & Samuelsson (1964) and performed as described by Downie, Poyser & Wunderlich (1974).

The prostaglandins extracted from the cow, bitch and ferret pyometrial fluid were then identified by combined gas chromatography and mass spectrometry (g.c.-m.s.) following the procedure of Thompson, Los & Horton (1970). Prostaglandins of the F series were first converted to the methyl ester, trimethylsilyl ether (Me-TMS) and identified in the manner outlined previously (Blatchley *et al.*, 1972). Prostaglandins of the E series were converted to the corresponding prostaglandin B compounds, reacted to form the Me-TMS derivatives, and then finally identified, by procedures described elsewhere (Downie *et al.*, 1974).

Prostaglandin F levels in ferret pyometrial fluid were also measured by radioimmunoassay (R.I.A.). A 100 μl portion of the fluid was diluted with an equal volume of 5% formic acid and extracted twice with 500 μl ethyl acetate. The organic phase extract was evaporated to dryness and the residue taken up in 200 μl tris-HCl buffer, pH 7.2. The concentration of prostaglandin F compounds was determined by the addition of 50 μl antiserum raised in a goat against prostaglandin $F_{2\alpha}$ conjugated to bovine serum albumin (titre used 1/500). This was followed by the addition of 50 μl buffer containing approximately 1000 ct/min $[^3H]$-prostaglandin $F_{1\alpha}$ (50 Ci/mmol) with incubation for 4 h at 4°C. The antibody-bound radioactivity was separated from unbound label by the addition of 300 μl saturated ammonium sulphate and 100 μl bovine gamma globulins (10 mg/ml buffer). The precipitate was separated by centrifugation and its radioactivity was determined by liquid scintillation spectrometry. The antiserum revealed a high cross-reaction with prostaglandin $F_{2\alpha}$ (100%) and prostaglandin $F_{1\alpha}$ (53.6%), but low cross-reactions with prostaglandins A_1, A_2, B_1, B_2, E_1, E_2, E_3 (all less than 0.04%).

Results

The results obtained in three cows, two bitches and one ferret are summarized in Table 1. Samples of pyometrial fluid were obtained from six uteri in amounts ranging from 17 to 110 ml. The fluid varied in consistency and nature, and was generally dark in colour. One sample of fluid from a bitch was quite viscous. The pyometrial sample obtained from a ferret had a thick consistency and a pungent odour. The pH of the fluid tended to be neutral or slightly alkaline, although the viscous sample from a bitch was slightly acid. The nature of the infection in the uterus was not determined and the cyclic activity and reproductive capabilities of the animals was not known except for one cow, which had a twin pregnancy.

The fluid from all six animals contained large quantities of prostaglandins. In all instances the prostaglandin F extracted was identified by g.c.-m.s. as being prostaglandin $F_{2\alpha}$. The prostaglandin E extracts consisted of predominantly prostaglandin E_2, though the extracts from two cow fluids were found to contain 5% to 10% prostaglandin E_1 as indicated by g.c.-m.s. Unfortunately, in two instances (one extract each from the cow and bitch), the amount of prostaglandin E-like material present was too small to obtain conclusive evidence of its identity by g.c.-m.s.

The levels of prostaglandin $F_{2\alpha}$ in the samples of cow and bitch pyometrial fluid, as measured by bioassay and uncorrected for procedural losses, ranged from 32 to 256 ng/ml. The level of prostaglandin E_2 was lower than the level of prostaglandin $F_{2\alpha}$ in each sample, and the levels varied from 6 to 85 ng/ml. The ratio of prostaglandin $F_{2\alpha}$ to E_2 ranged from 1.7 to 23.2. The concentration of prostaglandin $F_{2\alpha}$ in the pyometrial fluid of the ferret was 5.4 ng/ml, as determined by bioassay, and 10 ng/ml as measured by RIA. This difference is mainly attributable to

Table 1. Levels of prostaglandins in pyometrial fluid from the cow, bitch and ferret.

Species	*Sample No.*	*pH of fluid*	*Level of prostaglandin (ng/ml)* $PGF_{2\alpha}$	PGE_2	*Ratio of* $PGF_{2\alpha}:PGE_2$
Cow	1	7.0	142	85$^+$	1.7
	2	7.4	197	27$^+$	7.3
	3	–	32	6*	5.3
Bitch	4	5.6	256	11*	23.2
	5	7.2	117	56	2.1
Ferret	6	8.0	5.4	<0.5	–

$^+$ Contained 5 to 10% prostaglandin E_1 as indicated by combined gas chromatography and mass spectrometry (g.c.-m s.).

* Amounts present too low for positive identification by g.c.-m.s.

procedural losses which occurred during the former method of determination Prostaglandin E was not detectable (<0.5 ng/ml prostaglandin E_2) in the ferret pyometrial fluid, as measured by bioassay.

Discussion

This investigation has shown that pyometrial fluid obtained from the uterine lumen of both the cow and bitch contains high concentrations of prostaglandins $F_{2\alpha}$ and E_2. Prostaglandin $F_{2\alpha}$ was also found in pyometrial fluid from the ferret. Small amounts of prostaglandin E_1 were detectable in two samples of cow fluid. Thus, inflammatory fluid obtained from the uterus appears to contain prostaglandin $F_{2\alpha}$ predominantly, unlike inflammatory fluid from other sites which contains mainly prostaglandins of the E series.

In inflammation it is probable that tissue breakdown and the coalescence of cell contents results in a non-specific production of prostaglandins. In guinea-pigs and rats the uterus synthesizes mainly prostaglandin $F_{2\alpha}$ (Poyser, 1972; Williams, Sneddon & Harney, 1974), though it is not yet known whether this is true in the bitch, cow and ferret. It would appear, therefore, that inflammatory fluid contains significant amounts of the prostaglandin which the tissue is capable of producing, prostaglandin $F_{2\alpha}$ in the case of the uterus.

Pyometrial fluid from the cow and bitch was not lacking prostaglandin E_2, though the stimulus of prostaglandin production appeared incapable of directing synthesis towards producing predominantly prostaglandin E_2, the 'active' prostaglandin in inflammation (Beitch & Eakins, 1969, Crunkhorn & Willis, 1971). This is in contrast to the sheep where, under experimental physiological conditions, a sterile fluid containing only prostaglandin $F_{2\alpha}$ (the 'active' uterine prostaglandin in reproduction) collects in the uterus (Harrison, Heap, Horton & Poyser, 1972).

One cannot overlook the possibility that prostaglandins present in pyometrial fluid are formed in the uterus by cells other than uterine tissue cells themselves For example, the bacteria may be synthesizing and releasing the prostaglandins. In addition Higgs, McCall & Youlten (1975) have found phagocytosing polymorphonuclear leucocytes from the rabbit produce prostaglandins, though prostaglandin E_1 is the main one formed. It is likely that the majority of prostaglandins in pyometrial fluid are derived from the uterine tissue, though the prostaglandin E_1 found in two samples of cow pyometrial fluid could possibly have come from polymorphs.

It seems unlikely that uterine prostaglandins are the cause of infertility in animals suffering from pyometra. However, they may have a contributory role. Prostaglandin $F_{2\alpha}$ is released towards the end of the oestrous cycle in the cow, and it probably acts to cause regression of the corpus luteum in the ovary and thereby regulates oestrous cycle length (Nancarrow, Buckmaster, Chamley, Cox, Cumming, Cummins, Drinan, Findlay, Goding, Restall, Schneider & Thorburn, 1973). The present findings in the cow do present a paradox, since in this species the corpus luteum is sensitive to the luteolytic properties of prostaglandin $F_{2\alpha}$ and its analogues, yet in the condition of pyometra the life of the corpus luteum may be prolonged even though the luminal fluid in the uterus contains large amounts of prostaglandin $F_{2\alpha}$. This condition is similar to that produced experimentally in sheep by repeated daily doses of progesterone, which leads to a hydrometra rich in prostaglandin $F_{2\alpha}$. The prostaglandin $F_{2\alpha}$, is not released into uterine vein blood in significant amounts in the absence of oestrogen stimulation (Harrison & Heap, 1975). The general involvement of uterine prostaglandins in the control of fertility in primate and many sub-primate species is now under investigation.

This work was supported by a grant from the M.R.C. Authentic prostaglandins and prostaglandin $F_{2\alpha}$ antiserum were kindly supplied by the Upjohn Company, Kalamazoo, Michigan, U.S.A., and pyometrial samples by Mr A.D. Forteath of the Milk Marketing Board, Exeter, Devon, Mr J. Hammond Jr., Department of Applied Biology, University of Cambridge and Mr J. Hadley, The Royal Veterinary College, Potters Bar, Herts.

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(Received May 1, 1975.
Revised June 26, 1975.)

Br. J. Pharmac. (1975), 55 519-526

VENTRAL ROOT RESPONSES OF THE HEMISECTED AMPHIBIAN SPINAL CORD TO PERFUSED AMINO ACIDS IN THE PRESENCE OF PROCAINE

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1 The use of the procaine-blocked hemisected spinal cord preparation to identify the primary action of amino acids and their antagonists on amphibian motoneurones is described.

2 Apart from an anomalous effect of glycine, the responses of frog spinal motoneurones to amino acids were shown to be similar to those of mammalian spinal neurones.

3 In the presence of procaine, γ-aminobutyrate (GABA), taurine and β-alanine caused a hyperpolarizing response, measured in ventral roots, whereas L-glutamate and, to a lesser extent, glycine caused depolarization.

4 Picrotoxin and bicuculline specifically blocked ventral root responses to GABA, strychnine blocked responses to taurine and β-alanine but not responses to L-glutamate, glycine or GABA.

Introduction

Putative amino acid transmitters have been shown to have characteristic effects on the electrical activity of the hemisected amphibian spinal cord preparation as recorded from ventral and dorsal roots (Curtis, Phillis & Watkins, 1961). However, these actions of superfused amino acids on ventral root activity are complicated by indirect effects arising through actions on neurones which synapse with motoneurones or which are part of complex pathways terminating on motoneurones. To eliminate such indirectly mediated effects we have superfused preparations with procaine (1 mM) thereby eliminating all regenerative activity within the hemicord. The results show that, under these conditions, direct comparison can be made of the primary response of motoneurones to amino acids and of the potency of amino acid antagonists. In the course of the investigation, the use of procaine was compared with that of tetrodotoxin (Konishi & Otsuka, 1974) and high magnesium ion concentrations (Barker & Nicoll, 1973) both of which achieve a similar result. The preparation is discussed in terms of its usefulness for predicting amino acid agonist and antagonist activity on mammalian central nervous tissue.

Methods

Preparation of hemicords

Hemicords were set up in a superfusion trough as described by Curtis *et al.* (1961). Most experiments were carried out with hemicords from *Rana temporaria* but in some experiments hemicords from *Bufo bufo* were used. The latter preparations were somewhat easier to handle because of the longer spinal roots, but responses were similar in the two species, and frogs were more readily available. After being hemisected and placed in perfusion medium at 4° C, the preparations were found to be active for up to 8 days. Preparations were normally used within 4 days.

Electrical recording

Ventral roots VIII or IX were passed through a mixture of liquid paraffin/petroleum jelly and placed in contact with a chlorided silver wire. Potentials were recorded between this electrode and a similar one placed in contact with the Ringer solution. The perfusion medium was earthed via another Ag/AgCl electrode. Slow records of potential changes were made with a Vitatron 1 mV single channel ink writing pen recorder. Faster records of activity in ventral roots were made on photographic paper with a Telford oscilloscope camera and a Tectronix 502A oscilloscope. The records shown are representative of observations on at least 3 hemicords. An upward deflection on the records indicates an increase in negativity of the proximal region of the ventral root.

To stimulate the preparation, dorsal roots VIII or IX were passed through liquid paraffin/petroleum jelly and placed in contact with bipolar silver electrodes through which were passed supramaximal square wave pulses (50 V, 0.05 ms duration).

Composition of perfusion medium

The preparations were superfused at a rate of approximately 5 ml/min with a solution of the following composition (mM): NaCl 111, KCl 2, NaH_2PO_4 1, $CaCl_2$ 2, $NaHCO_3$ 10, glucose 12, tris base 10. The pH of this mixture was adjusted to 7.9 with 11.3 M HCl and the preparation was maintained at 12.5 ± 0.2° C. This solution was found to buffer adequately all the drug and amino acid solutions which were used; oxygenation was found to be unnecessary.

Addition of amino acid and drug solutions

The preparations were superfused at constant rate by means of a Watson Marlow MHRE 22 flow inducer. Amino acids and other drugs were dissolved in perfusion medium and placed in sample bottles in the rack of a Unicam SP4OP sample applicator. These solutions were added to the preparation by means of automatic pinchcocks which halted the flow of perfusion medium and allowed the drug solutions to flow onto the preparation. The period of perfusion of added substances was 40 s except where otherwise indicated.

Results

Ventral root responses of unblocked hemicords

In the absence of procaine, ventral root responses to perfused amino acids were generally similar to those previously reported (Curtis *et al.*, 1961; Tebēcis & Phillis, 1969; Barker & Nicoll, 1973). L-Glutamate invariably caused depolarization of motoneurones (Figures 2 and 3) accompanied by a burst of repetitive firing of ventral root fibres during the early stages of the action (Figure 1). The action of γ-aminobutyrate (GABA) was biphasic. An initial depolarization, which is particularly marked in Figure 3, was associated with repetitive spike discharges similar to those produced by L-glutamate (Figure 1) and this was succeeded by a hyperpolarization. At high doses of GABA the initial depolarization was not observed. Taurine and β-alanine (Figure 2) also produced hyperpolarizing changes without, however, any preceding depolarizing phases. Glycine (Figures 2 and 3), at the same concentration as those which produced easily measurable effects with the other three amino acids, often had no effect, but at higher doses invariably caused depolarization.

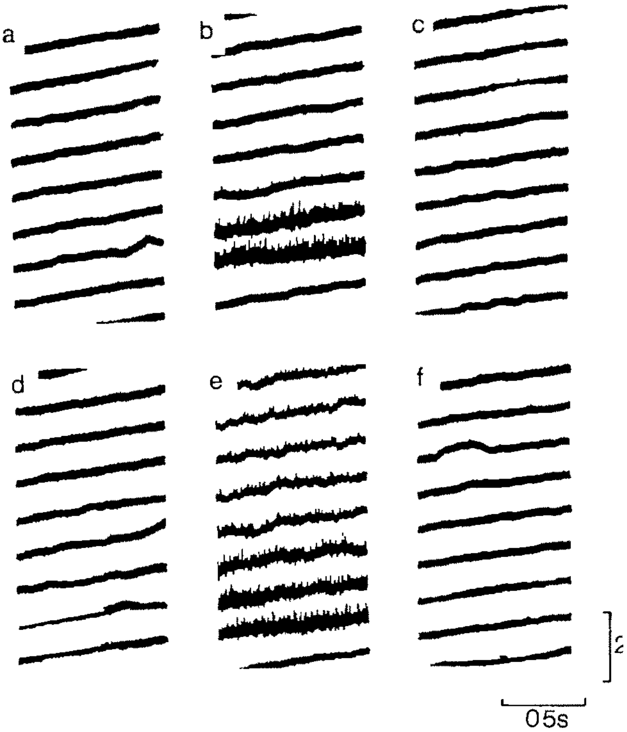

Figure 1 Effect of GABA (0.25 mM) and L-glutamate (0.5 mM) on spontaneous activity in ventral root of hemisected frog spinal cord. (a) and (c), 1 min before and 3 min after perfusion of L-glutamate respectively. (b) Recording during perfusion of L-glutamate (peak depolarization 0.42 mV) (e) Recording during perfusion of GABA (peak depolarization 1.17 mV). (d) and (f) 1 min before and 3 min after perfusion of GABA respectively. Each d.c. record progresses from the bottom to the top. Both amino acids were applied for a period of 45 seconds.

Ventral root responses of procaine-blocked hemicords

In the presence of procaine the responses measured in the ventral root to GABA, β-alanine and taurine were always hyperpolarizing and the responses to L-glutamate were always depolarizing (Figure 2). The responses to glycine were invariably depolarizing under the standard conditions described (Figure 2) but could be converted to a hyperpolarization by a variety of means; this will form the basis of a separate communication.

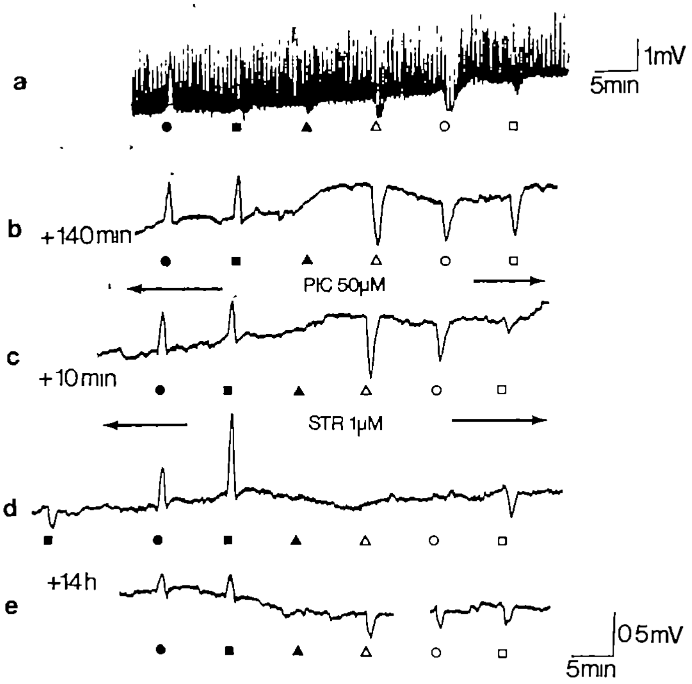

Figure 2 Comparison of the effects of amino acids on ventral root responses. (a) In the absence of procaine; (b)-(e) in the presence of procaine (1 mM); (c) effect of picrotoxin (PIC, 50 μM); (d) effect of strychnine (STR, 1 μM). Continuous d.c. record. L-glutamate, 0.25 mM (•); glycine, 1 mM (▪), α-alanine, 1 mM (▲), β-alanine, 0.5 mM (△), taurine, 0.5 mM (○), GABA, 0.5 mM (□).

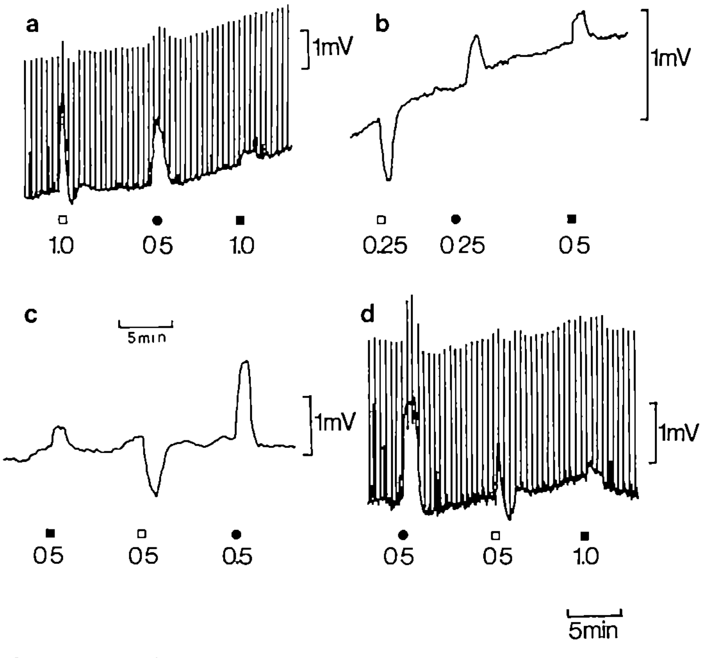

Figure 3 Effect of procaine on the ventral root response to amino acids of the toad spinal cord (d.c. record). Dorsal root stimulated at 2/minute. (a) Before addition of procaine to perfusion medium; (b) 100 min after adding procaine (1 mM) to perfusion medium; (c) another preparation in the presence of procaine (1 mM); (d) same preparation as (c), 100 min after washout of procaine. Glycine (▪); GABA (□), glutamate (•). Amino acid concentration (mM) indicated on record.

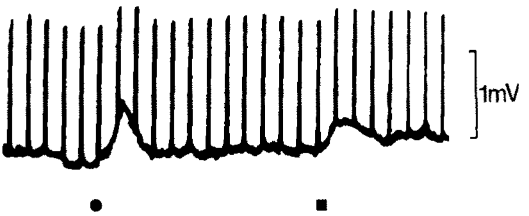

Figure 4 Effect of L-glutamate, 2.5 mM (●) and glycine, 10 mM (■) on the polarity of frog ventral root (L VIII) measured *in vivo*. The exposed spinal cord of the cerebrally pithed animal was superfused at 1.5 ml/min and at the symbols the flow of the perfusion medium was interrupted and amino acid solution was superfused for 40 seconds. Dorsal root stimulated 2/minute.

The depressant effect of procaine on the electrical activity of hemicords was reversible as shown by Figure 3 in which the actions of L-glutamate, GABA and glycine are compared. These effects of the three amino acids are typical of potential changes which were observed in over 30 preparations. The finding that glycine depolarized preparations on which GABA had a hyperpolarizing action contrasts with the known hyperpolarizing action of glycine on mammalian motoneurones (Werman, Davidoff & Aprison, 1968, and Curtis, Hösli, Johnston & Johnston, 1968). Glycine also had a depolarizing action when it was applied to amphibian spinal cords *in vivo* (Figure 4) and this indicates that it was unlikely to have been the *in vitro* experimental conditions which caused glycine to produce the observed depolarization.

Effects of amino acids on the polarization of ventral roots were found to be dose-dependent. Figure 5 shows a dose-response plot obtained for L-glutamate. It can be seen that the dose-response relationship is shifted to the right on treatment of the preparation with procaine, as would be expected from the depression of the indirect excitation of motoneurones by the superfused glutamate.

Procaine has a depressant action on conductance changes at the post-junctional membrane of frog skeletal muscle (Maeno, 1966). To determine if procaine had any such effects on amino acid responses apart from those due to blockade of regenerative activity, the action of amino acids was observed on preparations which were treated with tetrodotoxin (10^{-7} M) or MgSO$_4$(20 mM). Tetrodotoxin has no effect either on post-junctional conductance changes at the frog neuromuscular junction (Katz & Miledi, 1967) or on glutamate-induced depolarization of cat spinal motoneurones (Curtis, Duggan, Felix, Johnston, Tebēcis & Watkins, 1972). In the present experiments, the effect of tetrodotoxin and procaine were compared by measurement of the decrease in response to a standard dose of glutamate which occurred in the presence of these agents. A mean response of 58% (s.d. = 6) of the control response occurred in 5 tissues treated with tetrodotoxin (10^{-7} M); this compares with a mean value of 49% (s.d. = 14) for 7 tissues which were treated with procaine (1 mM). Amino acid responses obtained in the presence of MgSO$_4$ were similar to those obtained in the presence of procaine or tetrodotoxin. However, whereas procaine caused no depression of responses to L-glutamate in preparations which were treated with either tetrodotoxin or high Mg^{++} concentration, addition of MgSO$_4$ (20 mM) to procaine-blocked hemicords caused slight depression of responses to L-glutamate (Figure 6). In this experiment the control perfusion solution was made iso-osmotic with the MgSO$_4$ solution by the incorporation of sucrose (40 mM) into the control solution.

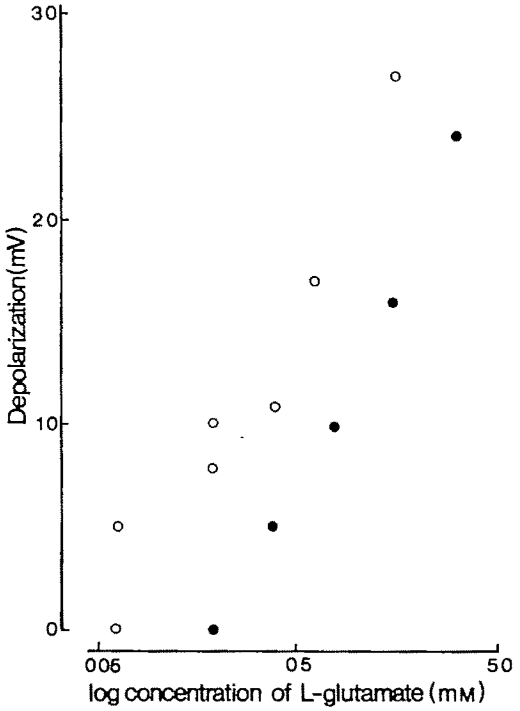

Figure 5 Relationship of concentration of L-glutamate to depolarization measured in ventral root of hemisected frog spinal cord. (○) Normal Ringer (procaine-free); (●) in the presence of procaine (1 mM).

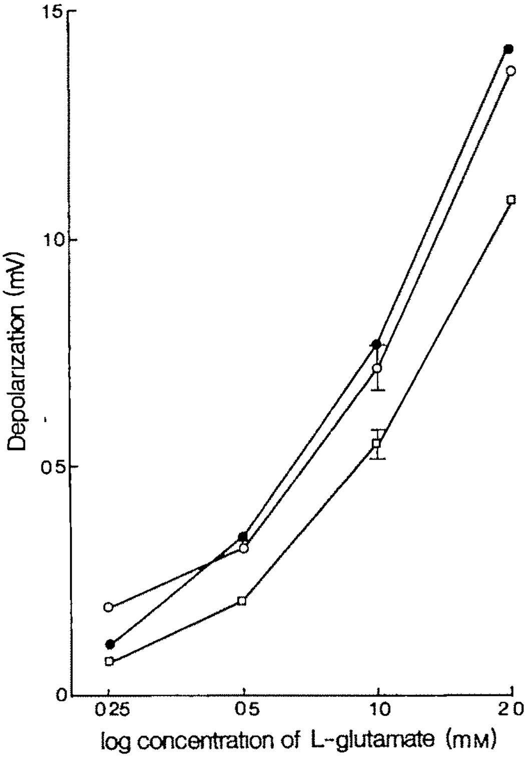

Figure 6 Depressant effect of $MgSO_4$ (20 mM) on ventral root response to L-glutamate in a procaine-blocked hemicord. (○) Control (+ 40 mM sucrose); (□) + 20 mM $MgSO_4$. (●) after washout of $MgSO_4$. Bars at 1 mM represent s.e. mean of 5 determinations.

It was of interest that the excitatory effects of acetylcholine (ACh) and carbachol, observed in unblocked preparations, were abolished by procaine (1 mM, Figure 7). This effect was not likely to have been caused by cholinolytic properties of the procaine because similar effects were also seen in preparations which were perfused with tetrodotoxin (10^{-7} M) or $MgSO_4$ (20 mM). As mentioned above, tetrodotoxin does not interfere with the local action of ACh at the neuromuscular junction (Katz & Miledi, 1967). Thus the excitatory action of ACh and carbachol on ventral root activity is most likely produced by an indirect action and not through stimulation of receptors on motoneurones. This conclusion contrasts with that of Matsuura (1971) who claimed the existence of depolarizing cholinoceptive sites on toad motoneurones. However, the experimental conditions used in that study may not have completely eliminated indirectly mediated effects.

Amino acid antagonists

Effects of strychnine and picrotoxin on ventral root potentials of the unblocked amphibian spinal cord have been reported previously (Tebēcis & Phillis, 1969; Barker & Nicoll, 1973; Barker, Nicoll & Padjen, 1975). The following results show that antagonism of amino acid induced changes in polarity of ventral roots may be specifically demonstrated in the presence of procaine.

Picrotoxin antagonized the responses produced by GABA but not those produced by L-glutamate, glycine, β-alanine or taurine (Figure 2c). The

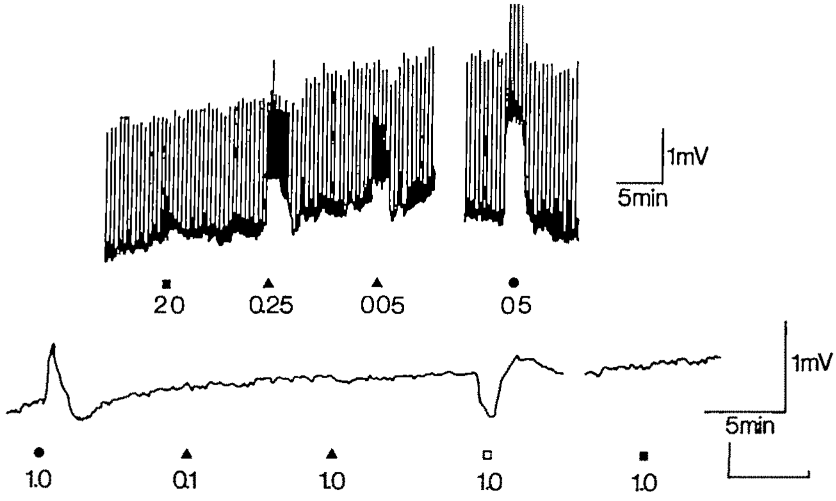

Figure 7 Effect of procaine (1 mM) on response of hemisected frog spinal cord measured in the ventral root (d.c. record) to acetylcholine (■); carbachol (▲); glutamate (●); GABA (□). Amino acid concentration (mM) indicated on record. Dorsal root stimulated at 2/minute. Lower trace in the presence of procaine (1 mM).

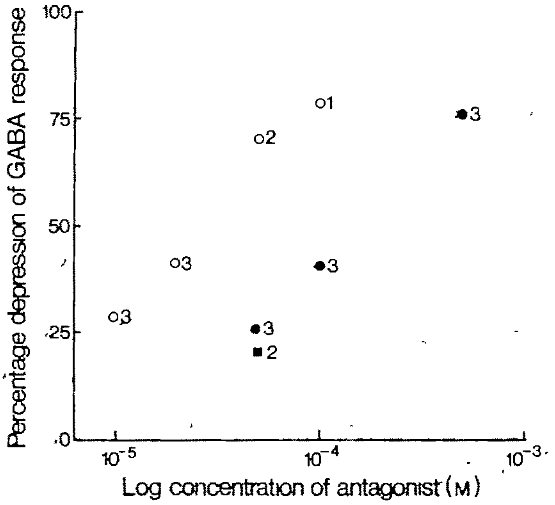

Figure 8 Comparison of picrotoxin (o), N-methyl bicuculline (●) and bicuculline (■) as antagonists of GABA in the procaine-blocked hemicord. The depression of the hyperpolarizing effect of 1mM GABA by each concentration of antagonist was measured in the ventral root. The antagonists were in contact with the preparation 5 min before addition of the test dose of GABA. The numbers suffixed to the points represent different preparations.

responses produced by taurine and β-alanine were blocked by strychnine but those produced by L-glutamate, glycine and GABA were not (Figure 2d). It should be noted here that strychnine, even in a 1 mM concentration, had no depressant effect on responses to glycine; indeed a potentiation was observed. The blockade of β-alanine and taurine responses by strychnine was much more slowly reversible than the antagonism of GABA responses by picrotoxin. Recovery from the effect of picrotoxin was usually complete after 3 hours. In the record shown in Figure 2 recovery from picrotoxin was incomplete at the time of the strychnine addition, but continued during the presence of the alkaloid. Little or no recovery of β-alanine and taurine responses was observed 3 h after washout of strychnine from hemicords. The preparation illustrated by Figure 2 was left overnight in strychnine-free medium, after which responses to β-alanine and taurine could once more be observed; at this stage, however, all the responses were somewhat attenuated.

With the method described it proved possible to compare the potencies of picrotoxin, N-methylbicuculline and bicuculline as GABA antagonists. This comparison is shown on Figure 8. In these experiments the depression of the hyperpolarization produced by a standard dose of GABA, in this case 1 mM, was measured in the presence of each concentration of antagonist. It can be seen from Figure 8 that picrotoxin was about five times more potent than bicuculline or N-methylbicuculline. Bicuculline and N-methylbicuculline appeared to be equipotent except that it was not possible to obtain accurate figures for bicuculline at concentrations higher than 0.1 mM because of its low solubility.

In the presence of procaine a saturated solution of bicuculline had no effect on ventral root responses to 1 mM glycine or L-glutamate, but depressed the response to 1 mM GABA by approximately 70%.

Discussion

It is clear from the results that, provided all regenerative activity is blocked, the isolated amphibian spinal cord preparation represents a useful system for the quantitative examination of the pharmacology of neuronal amino acid receptors. Other investigations have indicated, however, that amino acid receptors on different types of preparation may differ considerably from one another. For example, the relationship between structure and activity of excitatory substances at the insect neuromuscular junction (Clements & May, 1974) is very different from that observed on central neurones of mammalian preparations (Curtis & Watkins, 1960, 1963). The question therefore arises as to whether the amphibian preparation can provide experimental data of direct relevance to mammalian systems.

Recent results have indicated that, in the case of excitant amino acids, there is a remarkable degree of parallelism between excitation of rat spinal neurones and depolarization of the procaine-blocked frog spinal cord (Biscoe, Evans, Headley, Martin & Watkins, 1975, Evans & Watkins, 1975). This suggests a similarity in receptor structure for the excitatory amino acids in amphibians and mammals.

The only apparent exception to a similar parallelism between the action of inhibitory amino acids at amphibian and mammalian neurones is the effect of glycine. Whereas glycine hyperpolarizes cat spinal motoneurones, and strychnine blocks this action (Curtis *et al.*, 1968; Curtis, Hösli & Johnston, 1968), glycine depolarizes frog motoneurones and strychnine does not antagonize this response. On the other hand, strychnine does block specifically the hyperpolarizing effects of β-alanine and taurine on frog motoneurones, which parallels the antagonistic action of strychnine on both the hyperpolarization of cat spinal neurones by β-alanine and the depression of the firing frequency of cat spinal neurones by β-alanine and taurine (Curtis *et al.*, 1968; Curtis *et al.*, 1968). Moreover, the action of GABA is specifically blocked by picrotoxin and bicuculline in mammals (Galindo, 1969. Curtis, Duggan, Felix & Johnston, 1971, respectively) and amphibians. Further work will be required to determine if any other anomalies exist, but from the present evidence it would appear that many responses of frog motoneurones will be directly relevant to mammalian systems.

The specificity of picrotoxin as an antagonist of ventral root responses to GABA (Figure 2c) contrasts with the action of this agent on dorsal root responses. Barker *et al.* (1975) found that picrotoxin was equally effective as an antagonist of dorsal root depolarizations produced by taurine, β-alanine or GABA. Thus it would seem that receptors for taurine, β-alanine and GABA on dorsal root fibres and primary afferent terminals are less specific than those of motoneurones.

The difficulties of interpretation of both the site and type of action of substances applied to unblocked tissue is exemplified by the variability in the effects of inhibitory amino acids found by other investigators (Tebēcis & Phillis, 1969). A striking example obtained in the present work was the initial excitation caused by GABA (Figure 1). This effect may be explained by an initial inhibition by GABA of interneurones which exert a tonic inhibitory influence on frog motoneurones. Alternatively, intense primary afferent depolarization produced by GABA may cause the release of excitatory transmitter (Barker & Nicoll, 1973) onto interneurones which synaptically excite motoneurones. The blockade of regenerative activity within the cord by procaine abolished this excitatory phase and revealed the direct hyperpolarizing action of GABA on motoneurones.

Glycine is now generally accepted to be the strongest candidate for post-synaptic inhibitory transmitter function in the mammalian spinal cord, although the evidence does not exclude taurine and β-alanine from playing similar roles (Curtis & Johnston, 1974). The inhibitory effects of all three amino acids are antagonized by strychnine, which also blocks short latency post-synaptic inhibition of mammalian spinal motoneurones. If strychnine also blocks post-synaptic inhibition of amphibian spinal motoneurones, then the present results would indicate that the transmitter(s) involved are more likely to be taurine and/or β-alanine, rather than glycine. However, it should be noted that strychnine potentiated the glycine-induced depolarizations of frog motoneurones (Figure 2). This observation suggests the presence of a hyperpolarizing component of the glycine action which is sensitive to strychnine, and this component may represent a glycine action on inhibitory transmitter receptors.

Moreover, the possibility cannot be excluded that GABA also acts as a post-synaptic inhibitory transmitter at motoneurones. For instance bicuculline blocks the inhibitory effects of GABA at mammalian motoneurones (Curtis *et al.*, 1971) and the present results have shown that picrotoxin and bicuculline can antagonize the hyperpolarizing effect of GABA at amphibian motoneurones. The observation of a picrotoxin sensitive component of post-synaptic inhibition at cat motoneurones (Kellerth & Szumski, 1966) supports the possibility of a post-synaptic inhibitory transmitter role for GABA in mammals. However, a picrotoxin or bicuculline-sensitive post-synaptic inhibition of motoneurones in amphibians has yet to be demonstrated.

The authors are grateful for grants received from the Medical Research Council and the National Fund for Research into Crippling Diseases.

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(Received May 1, 1975.)

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THE EFFECTS OF LITHIUM IONS ON THE ANTIDIURETIC ACTION OF VASOPRESSIN IN THE RAT

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1 The effect of intravenous infusion of lithium, 2.56 μmol/min on the antidiuretic responses to antidiuretic hormone (ADH), adenosine triphosphate (ATP), 3′-5′ adenosine cyclic monophosphate (cyclic AMP) and theophylline was studied in water-loaded, alcohol-anaesthetized rats.

2 Lithium reversibly inhibits the antidiuretic response to all concentrations of ADH, depressing the maximum response but not changing the amount required for half maximal response.

3 The rate of increase of serum lithium relates more clearly to the inhibitory effect than does the serum concentration.

4 Sodium concentrations in the renal papilla seem to fall when serum lithium levels are rising.

5 Lithium inhibits the antidiuretic response to ATP and cyclic AMP but does not inhibit the response to theophylline.

Introduction

The polyuria produced by lithium salts in the treatment of affective disorders is of interest for several reasons. In some patients treatment has to be abandoned because a nephrogenic diabetes insipidus syndrome develops (Angrist, Gershon, Levitan & Blumberg, 1970). The prolonged administration of lithium also leads to a compensatory increased production of vasopressin (Jenner & MacNeil, 1974) which could explain behavioural effects of lithium in animals (Thompsen & de Weid, 1973). The inhibition is also of interest as elucidation of the mechanisms involved could lead to better understanding of renal physiology.

Polyuria produced by lithium results from the inhibition of the kidney's sensitivity to vasopressin (Thomsen, 1970; Forrest, Cohen, Torretti, Himmelhoch & Epstein, 1974). This is probably due to inhibition of renal vasopressin-sensitive adenyl cyclase (Dousa & Hechter, 1970). Lithium also inhibits many other adenyl cyclases which may lead to compensatory rises of other hormones and neurotransmitters. This could be of therapeutic relevance.

However, aspects of the mode of action of lithium on the kidney are disputed. Harris & Jenner (1972) demonstrated that intravenous infusion of lithium inhibited the antidiuretic response of the water-loaded alcohol-anaesthetized rat to Pitressin. This inhibition began after 10 minutes. It ceased quickly if the infusion no longer contained lithium, and the normal antidiuretic response was obtained even when plasma levels of lithium were still higher than those which were initially required to produce marked inhibition. Torp-Pedersen & Thorn (1973), using essentially the same methods, failed to show any reversal of the inhibition.

This paper attempts to throw further light on that discrepancy and on the mode of action of lithium in the kidney.

Methods

The rat preparation used was that described by Harris & Jenner (1972). Male rats (Anglia Laboratory Animals, CFY Wistar, 0.12-0.15 kg) were anaesthetized with intravenous pentobarbitone sodium (Nembutal, Evans, 35 mg/kg). A tracheotomy was performed where necessary, and then the bladder and the right external jugular vein were cannulated. Animals were infused via the jugular vein at 0.2 ml/min with a hypo-osmotic solution containing 51 mmol/l sodium chloride, 92.7 mmol/l glucose and 2.5% v/v ethanol, which is modified from Czaczkes, Kleeman & Koenig (1964). This was the control solution and it

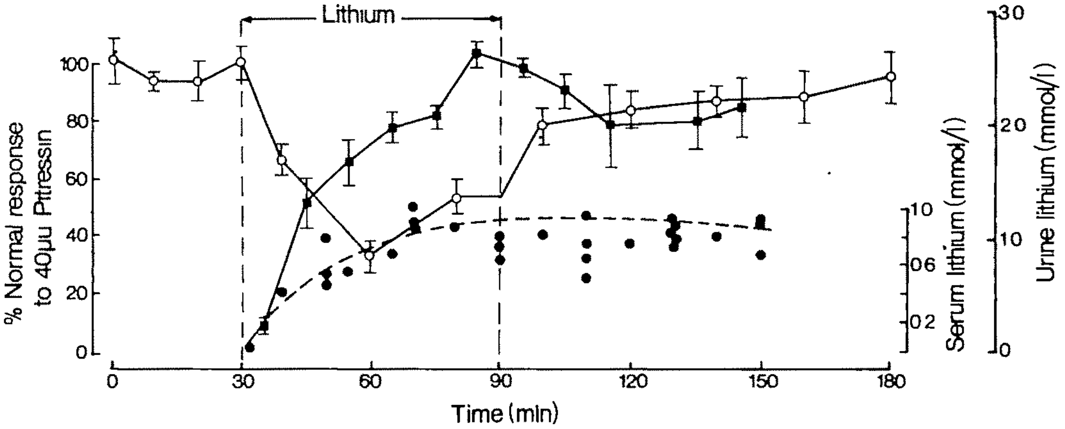

Figure 1 The antidiuretic responses to 40 μu Pitressin (o), expressed as a percentage of the 'normal' response, before, during and after a lithium infusion at 2.56 μmol/min for 60 minutes. Responses are shown at the moment of injection. The development and reversal of the inhibition can be noted. (■) Urine lithium and (●) serum lithium values from animals treated identically except for Pitressin injections. The lack of a correlation between these measurements and the inhibition of the response to Pitressin is apparent. Values for urine lithium and antidiuretic responses are presented as means; vertical lines show ± s.e. mean.

provided alcohol anaesthesia which, together with water-loading (approximately 8%), blocked the release of ADH. The control infusion was modified to form test lithium infusions by substituting lithium ions equimolarly for some of the sodium ions in the infusion. In one experiment 5 mmol/l theophylline (Aminophylline, Sigma Chemicals Company) was substituted for 5 mmol/l sodium in both control and lithium infusions. All infusions were given at 0.2 ml/minute. The normal rate of urine flow under control conditions was 0.17-0.2 ml/minute.

The antidiuretic response to Pitressin was calculated as the percentage reduction in urine volume in the 10 min after Pitressin compared to the 10 min before injection. To facilitate the presentation of responses from different groups of animals throughout a period of time, the antidiuretic responses were also expressed as a percentage of the 'normal' response to Pitressin, which was established for each animal.

Serial 10 min urine samples were collected in some experiments and in others terminal serum and kidney samples were taken after certain intervals of infusion. In the latter the abdomen was opened, the kidneys were clamped and a blood sample was collected from the cut carotid artery. The kidneys were removed and partially frozen to facilitate cutting. Both papillae and sections from cortices were taken. Material from both kidneys was combined and digested in an organic solubilizer, Soluene 100 (Packard Instruments, Ltd) for 24 hours. The digested sample was diluted with 2-ethoxyethanol to reduce viscosity.

Lithium in urine, serum and kidney tissue was estimated by atomic absorption spectroscopy. Sodium and potassium in serum and kidney were determined by flame photometry. Values are given as the mean ± s.e. mean and the significance of differences was calculated by Student's *t* test.

Results

Figure 1 shows the inhibition of the antidiuretic response to 40 μu Pitressin from 23 animals during the infusion of lithium at a rate of 2.56 μmol/minute. The lithium infusion lasted 60 min and was followed by the control infusion not containing lithium. Figure 1 also shows the serum lithium levels of 27 rats and the urinary lithium of 10 rats which received the same amount of lithium. These animals did not receive Pitressin. There is no evidence to suggest that Pitressin alters the distribution of lithium in the body, although it may temporarily reduce the rate of lithium excretion. It can be seen that inhibition of the activity of vasopressin is not related in a simple manner to either urine or serum lithium concentration. The serum level rises slowly and reaches a plateau. It falls slowly when the lithium infusion ceases, whereas the inhibition disappears quickly.

In Figure 2 the responses to 80 μu Pitressin of 32 rats are plotted against time. In these studies

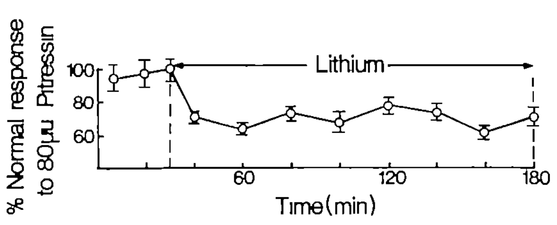

Figure 2 The antidiuretic responses to 80 µu Pitressin are shown before and during the infusion of lithium at 2.56 µmol/min for 150 minutes. Responses are shown at the moment of injection. The inhibition continues throughout the period of infusion The values are given as the means; vertical lines show ± s.e. mean.

the animals received the same infusion of lithium but for 150 minutes. Under these conditions the inhibition persists. In other words inhibition persists as long as the infusion contains a certain amount of lithium. Although not shown in Figure 2, the inhibition of the response to 80 µu Pitressin is also completely reversible after discontinuing the lithium. The length of the period of lithium infusion has not been found to affect the reversibility of the inhibition.

It will be noted in Figures 1 and 2 that the responses to vasopressin show greater inhibition after 30 min of infusion than after 50 minutes. This is a constant finding reaching statistical significance in Figure 1. It suggests an over-shoot. The results shown in Figure 1 imply a slightly slower reversal of the inhibition than that demonstrated by Harris & Jenner (1972) but they are in all other respects similar. They are consistent with the view that under these conditions a rising plasma lithium level causes inhibition which does not occur if the plasma level is dropping. The inhibition is independent of the actual lithium levels.

Figure 3 shows the renal concentration of lithium during experiments in which lithium 2.56 µmol/min was infused for 60 min, followed by the control infusion for the subsequent 60 minutes. The animals were killed sequentially at approximately 10 min intervals throughout the 60 min in which they received lithium and for the subsequent hour. The lithium levels in the kidney rose to a plateau and as can be seen both the cortical and the papillary concentration remained high after the lithium infusion ceased. Further the papilla to cortex ratio for lithium concentration did not change significantly during the experiment. In order to obtain figures for lithium concentrations in the kidney, large amounts of tissue were required and sections of both cortices and the whole of both papillae were pooled to make the estimations. Table 1 shows the sodium and potassium concentrations in the kidney sections for the animals used for Figure 3. The only significant finding is the difference

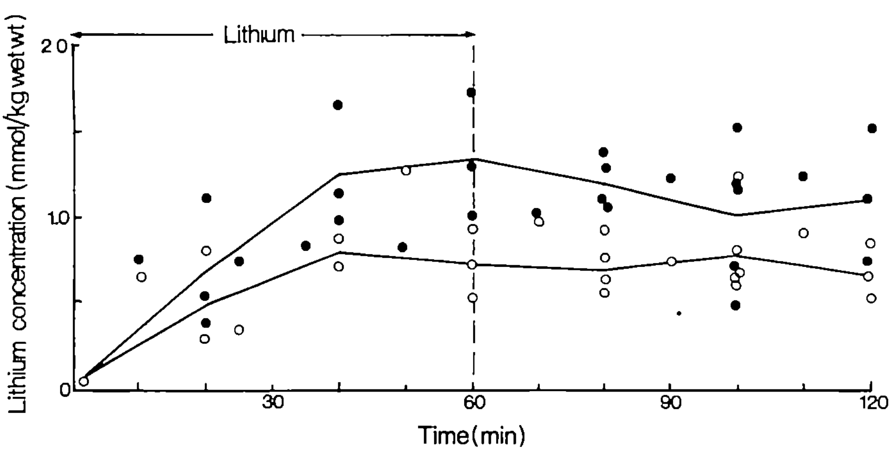

Figure 3 The increase in lithium concentration in the kidney is shown during the infusion of lithium (2.56 µmol/min), followed by 60 min of the control infusion. Lithium concentrations in the papilla (●) and cortex (o) of animals killed at various times throughout the experiment are shown Values are expressed as mmol/kg of tissue water. The lines shown on the graph join the mean lithium concentrations in the papilla and cortex at 20 min intervals throughout the experiment. Lithium concentration in the kidney remains quite high during the 60 min after lithium infusion, when (as shown in Figure 1), the inhibition of antidiuretic responses has ceased.

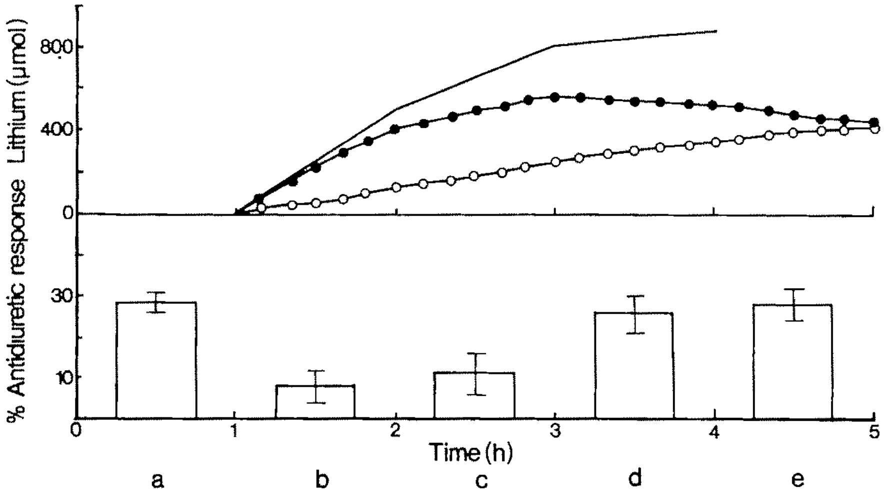

Figure 4 The effect of changing the rate of lithium entering the body on the antidiuretic response to Pitressin. The unbroken line shows the total amount of lithium given to the animal at any point in time. The amount of lithium excreted (o) is subtracted from the total lithium infused to give the amount of lithium present in the body (●). The lower portion of the figure shows the percentage antidiuretic response, i.e. percentage reduction in diuresis, due to 40 μu Pitressin during the infusion of solutions (a) to (e) for 60 min each. The values are mean ± s.e. mean of antidiuretic responses measured after 10, 30 and 50 min of infusion. The infusions contained.— (a) no lithium; (b) 8.6 μmol/min lithium; (c) 5.1 μmol/min lithium; (d) 1.0 μmol/min lithium; (e) no lithium. The rate of administration was constant throughout (0.2 ml/minute). The greatest reduction in the antidiuretic response is seen during the infusion of the highest lithium concentration, and as the rate of infusion of lithium ions decreases, the responses return to normal. Responses during (b) and (c) are significantly different from responses during (a); (d) is not significantly different from (a) or (e).

($P < 0.01$) between sodium concentrations in the papilla during and following lithium infusions.

Figure 4 shows results from experiments in which the antidiuretic responses to 40 μu Pitressin were measured under five different conditions. The normal responses of the animals are presented when they received no lithium during period (a), and then periods (b), (c) and (d) respectively show responses when 8.6, 5.1 and 1.0 μmol/min of lithium were infused. The final period (e) shows the normal response after discontinuing the lithium administration. The results are means from four animals. The lithium present in the body is also shown. It was calculated by subtracting that excreted in the urine from that infused. During the initial control period (a) with no lithium present,

Table 1 The concentration of sodium and potassium in renal tissue (mmol/kg of tissue water) and serum (mmol/l) during 60 min of lithium infusion (2.56 μmol/min) followed by 60 min of control infusion (no lithium)

	Sodium		*Potassium*	
	Lithium infusion (13)	*Control infusion (15)*	*Lithium infusion (13)*	*Control infusion (15)*
Cortex	48.6 ± 6.1	58.6 ± 3.8	70.0 ± 2.9	67.9 ± 1.7
Papilla	86.5 ± 4.0*	103.8 ± 4.9*	44.5 ± 1.5	50.9 ± 2.9
Means of P/C ratios	2.10 ± 0.21	1.86 ± 0.14	0.69 ± 0.15	0.80 ± 0.05
Serum	110.1 ± 1.9	104.9 ± 1.8	5.9 ± 0.1	6.2 ± 0.3

The animals were killed at 10 min intervals and the means of these values are presented. (n) = Total number of animals studied.

* The difference between these means is significant ($P < 0.01$).

the normal response to the 40 μu Pitressin was established (28 ± 2%). In the next hour (b) the lithium level in the body increased steeply and the antidiuretic response dropped to 8 ± 4%. During the third hour (c) the lithium level in the body continued to rise but at only approximately half the previous rate and the antidiuretic response increased. This increase was not statistically significant (11 ± 5%). In the fourth hour (d) the infusion contained a very low concentration of lithium and the lithium in the body actually dropped very slightly over the hour. The antidiuretic response during this latter period returned to 26 ± 5% which is not significantly different from the original response to the standard, 28 ± 2%, or to the response obtained in the subsequent hour (e) after stopping the lithium infusion when it was 28 ± 4%. In control experiments in which 12.8 mmol/l lithium infusion was given for up to 220 min, the concentration of lithium in the urine continued to increase during the period of infusion. In these experiments in which the rate of lithium entering the body decreased, the rate of lithium excretion also decreased. From Figure 4 it can be seen that the inhibition of the response to Pitressin does not follow the body content of lithium, but also that a situation can occur (d) in which despite high levels of lithium in the body fluids, lithium can still be given intravenously without producing significant inhibition of vasopressin.

During 60 min of 2.56 μmol/min lithium infusion, an animal receives approximately 0.15 mmol of lithium. When this same amount of lithium was given as one single acute injection, there was no inhibition of the action of 40 μu Pitressin given within 10-20 min after the injection of 0.15 mmol of lithium. Similarly 1.5 mmol lithium did not affect the response to 40 μu Pitressin measured 10-20 min after giving lithium. The lithium injection produced a diuresis for 10 min but this was similar to that produced by the injection of the same volume (1 ml) of saline. Pitressin given up to 100 min after the acute lithium injections in six rats also produced no inhibition of the antidiuretic response. In these experiments, therefore, a single injection of a quantity of lithium more than 10 times that normally required to produce inhibition of vasopressin in the infused animal, had no effect on the response to Pitressin a few minutes later.

The relationship between the rate of giving lithium and its action on the antidiuretic effect of Pitressin was further examined in 18 rats. Each animal was infused with lithium at two of three different rates. The order of infusion was varied so that some animals received high before low concentration lithium infusions and *vice versa.* Three animals also received control infusions between lithium solutions to check that the response to vasopressin had not changed during the experiment. As the order of administration of the lithium infusions did not seem to affect the responses, all the data were used (Figure 5). The mean inhibition of the normal response to 40 μu ADH over at least 60 min of infusion was calculated for each animal and from these responses a linear regression line was calculated which had a correlation coefficient $r = 0.80$ giving a P value of <0.001. The regression line is shown in the figure but clearly this response cannot be truly linear as the curve must (by definition) go through the origin and be asymptotic to 100% inhibition. The relationship is therefore more like an exponential or hyperbolic function and does approximate to % inhibition = $100(1-2^{-\text{rate}/12})$, also shown in the figure. The point which is to be emphasized is that the rate of administration of lithium is, under all the conditions studied, more useful in predicting percentage inhibition than concentrations of lithium in plasma, urine, whole body, or kidney.

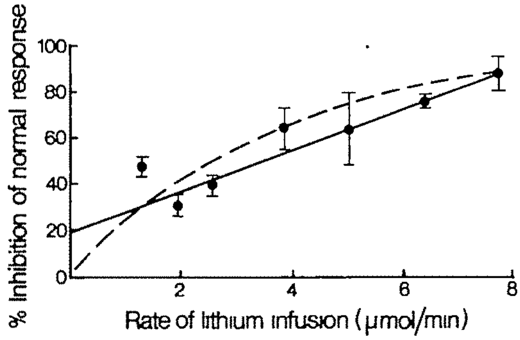

Figure 5 The relationship between the rate of lithium ions entering the body and the inhibition of the antidiuretic response to 40 μu Pitressin. Each response is expressed as a percentage inhibition of the normal response for that animal and the values are mean of antidiuretic responses measured after at least 10 min of infusion. Vertical lines show s.e. mean. A range of infusions in which lithium was substituted for sodium were given for at least 1 h to the animals. No animal received more than three infusions and the order of administering the infusions did not appear to affect the responses. The line shown passing through the data is a linear regression line, $r = 0.80$, $P < 0.001$. This statistic was used simply to indicate that there is a strong relationship between the extent of the inhibition and the rate of lithium entering the body. The figure also shows a dotted line which is an approximate exponential fit to the data.

In studies in which sodium and lithium concentrations were altered without regard for the

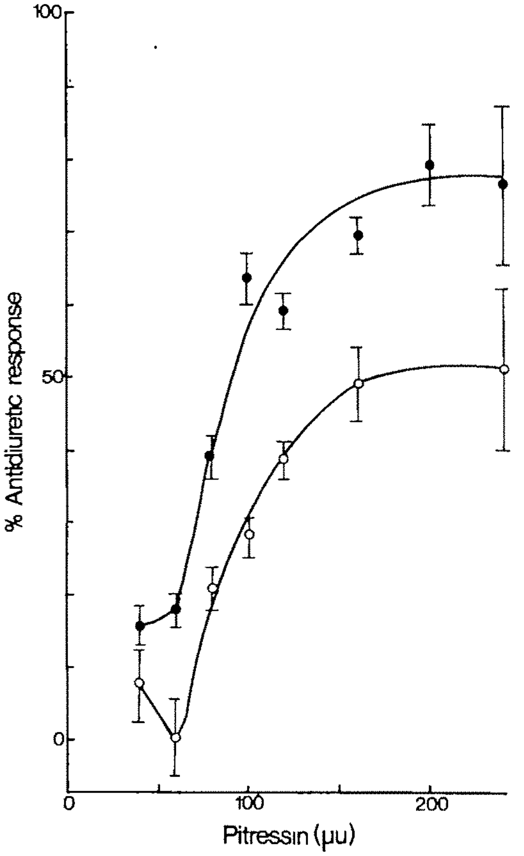

Figure 6 The antidiuretic response to a range of concentrations of Pitressin in the presence and absence of lithium The antidiuretic responses to a range of 40 to 240 μu Pitressin are shown (●) together with the responses during 2.56 μmol/min lithium infusion (○). The values are means of antidiuretic responses measured after at least 10 min of infusion. Vertical lines show s.e means. The maximum response to Pitressin is decreased in the presence of lithium, but the amount required for half the maximal response is unchanged.

osmolarity the ratio of sodium to lithium ions did not itself seem to determine the degree of inhibition of the action of Pitressin.

The above results suggest that direction of movement, or relative distribution of lithium across particular cells of membranes, might be more important than lithium concentration. However, Dousa & Hechter (1970), as well as others, have presented evidence that lithium inhibits the vasopressin-sensitive adenyl cyclase *in vitro*. To consider this further, the effect of lithium ions on the antidiuretic activity of adenosine triphosphate (ATP), 3′-5′adenosine cyclic monophosphate (cyclic AMP) and theophylline (an inhibitor of cyclic AMP phosphodiesterase), were explored in the anaesthetized rat. Table 2 summarizes the results from experiments in which 2 μmol ATP, 1:5 μmol cyclic AMP, or 15 μmol theophylline were injected intravenously during control and lithium infusions. The antidiuretic responses to ATP and theophylline had similar time courses to that produced by Pitressin, but the response to cyclic AMP took up to 60 min to develop. In one

Table 2 The inhibition by lithium of the antidiuretic responses to ATP, theophylline, cyclic AMP and Pitressin

	% Decrease in diuresis (x ± s.e. mean (n))			*Significance of differences between control and lithium responses*
Antidiuretic substance	*Control infusion*	*Lithium infusion*	*% Inhibition due to lithium*	P <
ATP (2 μmol)	34.3 ± 5.6 (17)	15.0 ± 5.5 (17)	56.3	0 001
Theophylline (15 μmol)	34.8 ± 5.1 (10)	35.6 ± 4.2 (11)	−2.2	NS
*Cyclic AMP (1 5 μmol)	57 ± 16.3 (10)	17.7 ± 6.2 (8)	65.8	0.01
Pitressin (30 μu)	31.2 ± 2.3 (14)	15.4 ± 2.7 (14)	50.6	0.001
	Control infusion + theophylline (5 mmol/l)	*Lithium infusion + theophylline (5 mmol/l)*		
Pitressin (30 μu)·	51.4 ± 6.2 (7)	25.1 ± 2 6 (12)	51.2	0.005

* The decrease in diuresis is measured by comparing the urine volume in the 10 min before the injection with the volume in the 10 min after the injection, except for the response to cyclic AMP, where the greatest reduction in the volume of urine, over a 10 min period, in the 60 min following the injection is compared to the volume of urine in the 10 min before the injection

experiment 5 mmol/l theophylline was added to both the control and lithium infusion to prevent breakdown of cyclic AMP within the renal tubular cell. From Table 2 it can be seen that lithium inhibited the antidiuretic response to ATP and to cyclic AMP but did not affect the response to theophylline. From other experiments the inhibition of ATP by lithium could not be overcome by increasing the amount of ATP given (i.e. up to 4 μmol). Lithium also inhibited the response to 30 μu Pitressin to the same extent (50%) whether theophylline was present in the infusion or not.

Figure 6 shows the effect of lithium on the antidiuretic responses to a range of Pitressin injections. The response to 40-240 μu Pitressin was determined during control and 2.56 μmol/min lithium infusions in 16 animals. It shows that lithium reduced the maximum response to ADH but did not significantly change the amount of hormone required for half maximal response. This suggests non-competitive inhibition by lithium for the site of action of the antidiuretic hormone.

Discussion

It is not possible to give a complete explanation of these findings. The results are consistent with the hypothesis that inhibition of the antidiuretic response to vasopressin is a function of the rate of increase of serum lithium concentrations. It is clear that reversal of the inhibition does occur. These results are compatible with those of Harris & Jenner (1972) but not those of Torp-Pedersen & Thorn (1973). Some renal mechanism readjusts to the inhibition, but there is probably a lag in this readjustment. As long as the lithium concentration is rising in the serum, the inhibition occurs because the adjustment is inadequate. When the concentration in the serum falls, the inhibition ceases.

However, the results presented are consistent with the view that lithium does inhibit sodium concentration by the counter-current system. Harris & Jenner (1972) showed a more precisely located reduction of sodium concentration in the inner medulla of the kidney when lithium was being infused. Looking at the data of Forrest *et al.* (1974), we note a very similar drop in concentration of sodium, but in what they term the outer medulla of the kidney of rats receiving lithium. In their report these changes are not statistically significant. Despite the fact that the different authors use different terminology, the areas in which they are reporting the sodium changes probably overlap. Solomon (1967), using large doses of lithium in dogs, with correspondingly high serum values of up to 15 mEq/l, has presented evidence implying that there are no changes in the distribution of sodium in the kidney during lithium treatment. Forrest *et al.* (1974) themselves also draw the same conclusion from their own results. We therefore must be cautious in stating that lithium can affect the overall handling of sodium in the countercurrent mechanism. Nevertheless this might be predicted on general principles. If lithium infusion does reduce the sodium concentration there would be a reduced osmotic pressure at the tip of the loop of Henle. The latter would decrease the ability of the kidney to form hypertonic urine in the collecting ducts. According to Dousa & Hechter (1970), the reduced sodium would also possibly increase the sensitivity of the adenyl cyclase to vasopressin. In other words the changes in sodium would have two opposite effects. The situation is however complicated by the inhibition of the adenyl cyclase by lithium.

Our speculations lead to the view that some internal mechanisms attempt to maintain sodium concentration and/or adenyl cyclase sensitivity in such a way as to stabilize the responses to the antidiuretic hormone. When lithium concentration is rising fast enough compensation lags.

The action of lithium on renal adenyl cyclase in the whole animal was investigated. The results are compatible with those obtained by Dousa (1974) in studies of human biopsy material. The adenyl cyclase activity in the rat bioassay preparations is basal as the endogenous release of ADH is thought to be inhibited. Lithium infusion alone does not change diuresis and it does not inhibit the antidiuretic response to theophylline; therefore lithium does not appear to affect the basal activity of the enzyme. Lithium inhibited the antidiuretic response to ADH over a range of doses of ADH, depressing the maximum response to ADH while not changing the amount of ADH required for half maximal response. These particular characteristics of the relationship of the two dose-response curves suggest non-competitive inhibition.

Lithium inhibition of ADH also occurred to the same extent in the presence or absence of theophylline, suggesting that this action of lithium is not dependent on the phosphodiesterase breakdown of cyclic AMP. These particular results in the whole animal agree with the detailed tissue study of Dousa (1974), in which he found lithium specifically inhibited the ADH-stimulated activity of adenyl cyclase but not the basal activity and that lithium did not affect the activity of cyclic AMP phosphodiesterase.

The inhibition of the response to ATP might be explained by inhibition of adenyl cyclase, but if so it is a little surprising that increased amounts of

ATP do not seem able to overcome the inhibition. In the case of cyclic AMP the inhibition due to lithium must be at a point beyond the formation of cyclic AMP. This inhibition of the action of cyclic AMP must play some part in all of the studies in this paper.

There seems to be little doubt that lithium does inhibit vasopressin-sensitive adenyl cyclase, and therefore that this does play an important role in the inhibition of the action of the antidiuretic hormone on the kidney. However, enzyme inhibition alone leaves the peculiar lack of relationship between serum lithium and the inhibitory effect of lithium unexplained. If the effect is dependent on adenyl cyclase, only lithium concentration at the particular time of inhibition should affect it. Clearly intracellular concentration rather than renal or serum lithium would be extremely interesting, and particularly the lithium concentration at the actual site of the enzyme, but this is impossible to study.

From the clinical point of view, acute inhibition in the kidney due to lithium is of little significance. The relevance of the acute animal studies to the chronic clinical situations can therefore be questioned. Lithium is only therapeutically effective after several days of treatment. Further, the lithium-induced polyria which occurs in man does not appear in all individuals taking lithium, and it takes a very variable length of time to develop, often running into months (Angrist *et al.*, 1970; Forrest *et al.*, 1974).

Geisler, Wraae & Oleson (1972) have presented evidence suggesting that over long periods of lithium administration the adenyl cyclase system itself has altered. Some long term readjustment in the body to the possible intermittent inhibition of vasopressin by lithium probably occurs in the kidney, as shown by Geisler *et al.* (1972) and also in the hypothalamus, which produces an increase in vasopressin release (Jenner & MacNeil, 1974).

S.McN. was an M.R.C. scholar.

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(Received May 21, 1975.
Revised July 1, 1975.)

Br. J. Pharmac. (1975), 55, 535-539

THE INFLUENCE OF METYRAPONE ON THE SYNTHESIS AND RELEASE OF PROSTAGLANDINS FROM THE PREGNANT RAT UTERUS *in vitro*

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1 Metyrapone (150 mg/kg, s.c. or i.p.) an inhibitor of corticosteroid biosynthesis, significantly reduced the release of prostaglandins of the F-type from isolated preparations of pregnant rat uteri *in vitro*, on day 22 – the expected day of delivery.

2 Metyrapone and indomethacin administered *in vitro* both inhibited the conversion of ^{14}C-arachidonic acid to prostaglandin E_2 by homogenates of day 22 pregnant rat uteri. Metyrapone was approximately 150 times less potent than indomethacin. Although indomethacin also inhibited prostaglandin $F_{2\alpha}$ production, metyrapone stimulated synthesis of this prostaglandin. The differential inhibition of prostaglandin synthesis by metyrapone may reflect sensitivity of the inhibitor to changes in experimental conditions.

3 Inhibition of prostaglandin synthesis may explain the effects of metyrapone on parturition in the rat.

Introduction

An increasing body of evidence appears to indicate a role of the foetal adrenal glands in the mechanism of the initiation of parturition in the sheep and other long-gestation species (see Liggins, Fairclough, Grieves, Kendall & Knox, 1973). In the rat, however, the role of the foetal hypophyseal-adrenal system is obscure. Foetal plasma and adrenal tissue corticosterone concentrations have been shown to increase towards the end of pregnancy, but fall during the last 2 days (Kalavsky, 1971; Cohen, 1973). Administration of metyrapone, an inhibitor of corticosteroid biosynthesis, causes foetal hypertrophy (Dupouy, 1972) and prolongs pregnancy in the rat (Parvez, Parvez & Roffi, 1972), though removal of the foetal brain did not alter gestation length (Swaab & Honnebier, 1973) and surgical removal of the foetuses is reported not to delay parturition in this species (Selye, Collip & Thomson, 1935; Kirsch, 1938). However, this latter procedure results in a significant reduction in the *in vitro* uterine release of prostaglandin F (Parnham, Sneddon & Williams, 1975). Prostaglandin F is released *in vitro* in increasing amount during the last 5 days of pregnancy in the rat, reaching a maximum on the expected day of delivery and this release is related to an increase in uterine activity *in vitro* (Vane & Williams, 1973; Harney, Sneddon & Williams, 1974; Williams, Sneddon & Harney, 1974). Administration of antibody to prostaglandin $F_{2\alpha}$ delayed parturition in the rat (Dunn, Humphries, Judkins, Kendall & Knight, 1973) and administration of indomethacin (Aiken, 1972; Chester, Dukes, Slater & Walpole, 1972), an inhibitor of prostaglandin biosynthesis (Vane, 1971), both reduced the release of prostaglandin F and delayed parturition in the rat.

The present study was carried out to investigate the effect of metyrapone on the release of prostaglandin F from the pregnant rat uterus *in vitro*. Some of these results have been demonstrated to the British Pharmacological Society (Parnham & Sneddon, 1975).

Methods

Mating

Porton strain virgin female rats 6-8 weeks old were mated singly overnight with proven males and successful matings were determined by the appearance of vaginal plugs the following morning (designated day 1). Housing conditions have been described previously (Parnham *et al.*, 1975).

Drug administration

For studies on *in vitro* prostaglandin F release pregnant rats were caged singly in two groups with nesting materials on day 19 of pregnancy. Group I· rats received daily injections of metyrapone (150 mg/kg, s.c. or i.p.). Group II rats received an equivalent volume of vehicle, 0.33 M (+)-tartaric acid (0.75 ml/kg, s.c. or i.p.). All injections were given between 16.00 h and 17.00 h on days 20 and 21 of pregnancy.

Isolated uteri

Three control and 4 metyrapone-treated rats were killed on the morning of day 22 by a blow on the head, the uteri removed, and individual horns mounted in a 75 ml organ bath as described by Vane & Williams (1973) Bath fluid was collected and extracted for prostaglandins as described previously (Vane & Williams, 1973).

Bioassay of extracts

Extracts were reconstituted with 0.9% NaCl solution and assayed on the rat isolated colon against authentic prostaglandin $F_{2\alpha}$ as described previously (Harney *et al.*, 1974).

Homogenization

Two untreated pregnant rats were killed on the morning of day 22 by a blow on the head, the uteri removed and immediately placed in ice-cold phosphate buffer, pH 7.5, containing (g/l) Na_2HPO_4 (14.2), and $NaH_2PO_4.2H_2O$ (15.6). The uteri were opened longitudinally and the foetuses and placentae removed. The tissue was then chopped and homogenized in 7 ml ice-cold phosphate buffer (pH 7.5), using an Ultra-Turrax homogenizer (2 x 10 s separated by 60 seconds).

Radiometric assay

This assay is a modification of the method of Blackwell, Flower, Parsons & Vane (1975). The incubation mixture (in 25 ml Ehrlenmeyer flasks) consisted of the following: 50 µl $[^{14}C]$-arachidonic acid in *n*-hexane (10-14,000 d/min) evaporated to dryness under nitrogen and resuspended, with shaking, in 1 ml phosphate buffer, pH 7.5; 1 µg prostaglandin $F_{2\alpha}$ (in 0.1 ml) and 1 µg prostaglandin E_2 (in 0.1 ml) to minimize metabolism of labelled product; 0.1 ml phosphate buffer (for boiled blank and control), 0.1 ml metyrapone (final concentrations 0.05 mM, 0.2 mM and 0.5 mM) or 0.1 ml indomethacin (final concentrations 0.001 mM, 0.002 mM and 0.005 mM), 1 ml homogenate (giving a final protein concentration of about 20 mgm). Protein determination was carried out by the method of Lowry, Rosebrough, Farr & Randall (1951). Each incubation flask was then shaken and incubated for 10 min in a shaking water bath at 36° C against a boiled blank. (Time course studies showed that both prostaglandin E_2 and $F_{2\alpha}$ synthesis reached 80% of maximum within 15 minutes) The reaction was stopped by pouring the incubates into centrifuge tubes, washing the flasks with 1 ml phosphate buffer and boiling on a water bath for 60 seconds. A further 1 ml buffer was added to each tube to cool down the contents, followed by mixing and centrifugation at 1000 *g* for 5 minutes. The supernatant was decanted, acidified to pH 3 with 1 N HCl, and 1 ml saturated NaCl solution was added to facilitate protein precipitation. This mixture was once again centrifuged at 1000 *g* for 5 minutes. The supernatant was then decanted and extracted twice with two volumes ethyl acetate. The two organic fractions were pooled, evaporated to dryness and resuspended in 1 ml 2% methanol in chloroform. (Alternatively the samples were stored at −21° C before being resuspended.) Each sample thus resuspended was subjected to column chromatography on 8 cm silicic acid (325 mesh) columns using 2%, 4% and 10% methanol in chloroform in 10 ml volumes as successive eluants (K.I. Williams, personal communication). Prostaglandin E_2 was collected in the 4% fraction and prostaglandin $F_{2\alpha}$ in the 10% fraction. Each fraction was evaporated to dryness, resuspended in scintillation fluid and counted on a Packard Tri-Carb liquid scintillation counter. Two experiments were carried out to check the purity of the column fractions by subjecting the three fractions obtained from control incubates and boiled blank to thin layer chromatography on 0.25 mm silicic acid plates using system E of Nugteren & Hazelhof (1973) against authentic prostaglandin E_2, D_2 and $F_{2\alpha}$. The plates were divided into 1 cm zones which were scraped into scintillation vials for counting.

Materials

Metyrapone was obtained from CIBA, Horsham; indomethacin from Merck, Sharpe & Dohme; $[^{14}C]$-arachidonic acid (specific activity 54 Ci $mmole^{-1}$); $[^3H]$-PGE_2 (specific activity 59 Ci $mmole^{-1}$) and $[^3H]$-$PGF_{2\alpha}$ (specific activity 59 Ci $mmole^{-1}$) from the Radiochemical Centre, Amersham; silicic acid, 325 mesh, from Sigma Chemicals, London, 0.25 mm silicic acid TLC plates (20 x 20 cm) from Merck.

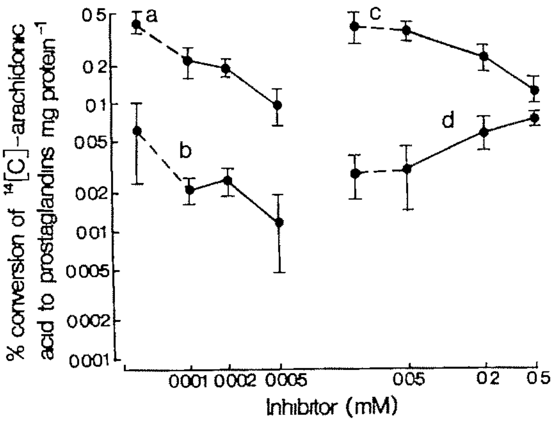

Figure 1 Effects of metyrapone and indomethacin on prostaglandin synthesis in the pregnant rat uterus *in vitro*. (a) and (b) show the effect of indomethacin on prostaglandin E_2 and prostaglandin $F_{2\alpha}$ synthesis respectively; (c) and (d) show the effect of metyrapone on prostaglandin E_2 and prostaglandin $F_{2\alpha}$ synthesis respectively. Mean control values for each dose-response curve are shown (± s.e. mean) connected by dotted lines. The results with indomethacin were obtained from 4 different uterine homogenates and the results with metyrapone from 3 different homogenates. Using smaller amounts of added [^{14}C]-arachidonic acid the actions of indomethacin were confirmed with a further 2 homogenates and the actions of metyrapone with a further 3 homogenates.

Results

Effect of metyrapone on prostaglandin F release and uterine activity in vitro

In rats treated with 150 mg/kg metyrapone on days 20 and 21 of pregnancy the mean prostaglandin F release over a 1 h period from single uterine horns *in vitro* determined on day 22 was 173.8 ± 21.2 (s.e mean) ng g^{-1} h^{-1} (7 horns). This was significantly less than the prostaglandin F release from control horns (253 ± 35.5 ng g^{-1} h^{-1}; 6 horns) ($P < 0.05$; Wilcoxon Test). Spontaneous contractions of the control horns were well maintained through the 1 h period *in vitro*, but in horns from metyrapone-treated rats activity declined after 30 min *in vitro*.

Validation of prostaglandin synthetase assay

Column chromatography of control incubations of [^{14}C]-arachidonic acid with pregnant rat uterine homogenates followed by thin layer chromatography demonstrated that the majority of the radioactivity recovered was distributed as follows arachidonic acid and neutral lipids in the first fraction, prostaglandin E_2 in the second fraction and prostaglandin $F_{2\alpha}$ in the third fraction. Nine to ten percent of the radioactivity originally added to control incubates as [^{14}C]-arachidonic acid was converted to prostaglandins. In one experiment pellets from centrifugation and all aqueous layers were counted and the remaining 90% of the radioactivity was accounted for as unchanged arachidonic acid, label taken up by tissue (probably into phospholipids) and water-soluble products. Recovery of [^{3}H]-prostaglandin E_2 (15,000 d/min) through extraction and chromatography was 65-70% and recovery of [^{3}H]-prostaglandin $F_{2\alpha}$ (60,000 d/min) was 55-65%.

Effects of metyrapone and indomethacin on prostaglandin synthesis in vitro

Metyrapone introduced *in vitro* inhibited prostaglandin E_2 synthesis in pregnant rat uterine homogenates in a dose-dependent manner. Indomethacin inhibited both prostaglandin E_2 synthesis (about 150 times more effectively than metyrapone) and prostaglandin $F_{2\alpha}$ synthesis (Figure 1).

Discussion

The results presented here show that metyrapone, an inhibitor of the 11β-hydroxylation step in the biosynthesis of corticosteroids (Chart & Sheppard, 1959) is also an inhibitor of prostaglandin synthesis, but has differential effects *in vivo* and *in vitro*. In the isolated pregnant rat uterus prostaglandin $F_{2\alpha}$ is the predominant prostaglandin released (Vane & Williams, 1973). This observation was confirmed in one experiment using column chromatography and bioassay against authentic prostaglandins. In this preparation inhibition of prostaglandin $F_{2\alpha}$ release from the uterus was observed after administration of metyrapone to pregnant rats. However, in homogenates prepared from pregnant rat uteri, prostaglandin E_2 was the predominant prostaglandin produced and the addition of metyrapone to the homogenate resulted in inhibition of prostaglandin E_2 synthesis but a paradoxical stimulation of prostaglandin $F_{2\alpha}$ synthesis. This effect of metyrapone was observed over a wide range of metyrapone concentrations and contrasted with indomethacin which inhibited the synthesis of both prostaglandins. We are unable to offer an explanation for the observation that the isolated uterus produces predominantly prostaglandin F when homogenates produce predominantly prostaglandin E. Preliminary experiments have shown that the balance of prostaglandin E and prostaglandin F synthesis in homogenates is unaffected by altering either substrate concentration or incubation time. However, it is pertinent to note that other workers have reported differential effects of inhibitors on prostaglandin synthesis, which are related to changes in conditions such as substrate concentration (Flower, Cheung & Cushman, 1973) or cofactors (Maddox, 1973). Similarly, the effect of metyrapone on prostaglandin synthesis in uterine homogenates may be sensitive to changes in experimental conditions. It is of interest to note that the structure of metyrapone (2-methyl-1,2-bis-(3-pyridyl)-1-propanone) is similar to those of many other prostaglandin synthetase inhibitors which also consist of two interconnected ring systems (Gryglewski, 1974).

Inhibition of prostaglandin synthesis may explain the prolongation of pregnancy produced by metyrapone in the rat (Parvez *et al.*, 1972), since several prostaglandin synthetase inhibitors have been shown to prolong and/or delay parturition in this species (see Aiken, 1974). It is unlikely that metyrapone affects parturition through inhibition of foetal adrenocorticosteroidogenesis, as there is little evidence for foetal involvement in parturition in the rat (Jost, 1973). However, ovarian oestrogen is implicated in parturition in the rat (Yoshinaga, Hawkins & Stocker, 1969) and as metyrapone inhibits oestrogen synthesis (Griffiths, 1963; Matsumoto, Kotoh, Miyata & Kurachi, 1965) this may, in part, explain its effects on parturition.

M.J.P. is an MRC Scholar. We thank Dr J.E. Pike of the Upjohn Company and Dr R.J. Flower of the Wellcome Research Laboratories for gifts of prostaglandins.

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(Received June 6, 1975.
Revised July 21, 1975.)

Br. J. Pharmac. (1975), 55, 541-546

ASSESSMENT IN THE GUINEA-PIG ILEUM AND MOUSE VAS DEFERENS OF BENZOMORPHANS WHICH HAVE STRONG ANTINOCICEPTIVE ACTIVITY BUT DO NOT SUBSTITUTE FOR MORPHINE IN THE DEPENDENT MONKEY

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1 Four benzomorphans which have potent antinociceptive activity in the hot-plate and writhing tests in the mouse but do not suppress or precipitate withdrawal symptoms in the morphine-dependent monkey, have been examined for their pharmacological actions in the guinea-pig ileum and mouse vas deferens.

2 In the guinea-pig ileum their agonist potencies are 1.5 to 400 times greater than that of normorphine or morphine whereas in the mouse vas deferens their potencies relative to morphine are 0.3 to 100. They exhibit no antagonist activity in either preparation. Benzomorphans which substitute for morphine in the morphine-dependent monkey do not show such differences between their relative potencies in the guinea-pig ileum and mouse vas deferens.

3 The relative potencies of the four benzomorphans to inhibit stereospecific [^{3}H]-dihydromorphine binding by membrane fragments from rat brain, are more closely related to their relative agonist potencies in the mouse vas deferens than to those found in the guinea-pig ileum.

4 In order to antagonize the agonist actions of these benzomorphans, naloxone is required in concentrations which are 3 to 7 times higher than those needed for the antagonism of normorphine or morphine or of benzomorphans which suppress abstinence in morphine-dependent monkeys.

5 It may be possible to use the three assays, namely, ratio of relative agonist potency in mouse vas deferens to that in guinea-pig ileum, ratio of relative agonist potency to relative affinity to opiate receptors and the concentration of naloxone required for antagonism, for the prediction of the potential of new compounds to produce physical dependence.

Introduction

In recent years, some benzomorphans related to cyclazocine have been shown to have unusual pharmacological properties. Two of these compounds are N-cyclopropylmethyl-5,9-dialkyl-2′-hydroxy-6,7-benzomorphans in which an oxo-group has been introduced at C_8 (Albertson, 1974). The other two compounds are N-dimethylfuryl-5,9-dimethyl-2′-hydroxy-6,7-benzomorphans (Merz, Langbein, Stockhaus, Walther & Wick, 1974). These compounds differ from other benzomorphans in that they have potent antinociceptive effects in the hot-plate test in mice but, rather unexpectedly, do not suppress the signs of morphine withdrawal in morphine-dependent monkeys (Villarreal & Seevers, 1972; Swain & Seevers, 1974). In tests on rodents, the compounds have either very little or no antagonist action (Archer, S. & Pierson, A.K., personal communication, 1972; Merz *et al.*, 1974) and in the morphine-dependent monkey, they do not cause a withdrawal syndrome (Villarreal & Seevers, 1972; Swain & Seevers, 1974).

In this paper, it will be shown that the normally close correlation between the relative agonist potencies found in the two preparations of the guinea-pig ileum and mouse vas deferens (Hughes, Kosterlitz & Leslie, 1975b) does not hold for these compounds. Furthermore, it has been found that higher concentrations of naloxone are required to antagonize the agonist effects of these compounds than those of morphine, codeine, levorphanol and other congeners of morphine. Some of the results have been presented to a Work Session of the M.I.T. Neurosciences Research Program, May 1974 (Kosterlitz, 1975).

Methods

Experimental procedures

The methods used are those described earlier (Kosterlitz & Watt, 1968; Hughes *et al.*, 1975b). In principle, segments of ileum were stimulated coaxially (0.1 Hz, 0.5 ms duration, supramaximal voltage) and the mouse vas deferens by field stimulation (0.1 Hz, 1 ms, supramaximal voltage). For the determination of the relative agonist potency, dose-response curves were constructed, the concentration which caused a depression of 50% of the electrically evoked contraction was compared to a concentration of normorphine which gave the same depression. Normorphine was chosen as standard of reference because the onset and offset of action was more rapid than that of morphine, with which normorphine is equiactive in both preparations. Relative agonist potencies were calculated for each assay as the ratio ID_{50} of normorphine/ID_{50} of test substance.

The antagonist potency of naloxone was obtained by constructing dose-response curves of the benzomorphans in the absence and in the presence of 3 or 4 concentrations of naloxone. From the dose-ratios obtained, regression lines were calculated for log (dose-ratio −1) on log naloxone concentration. With all but one of the compounds, the slope of the regression line did not deviate significantly from unity; the equilibrium dissociation constant (K_e) was calculated from the intersection of the regression lines with the abscissa scale and expressed in nM. K_e indicates the concentration of naloxone which causes a dose-ratio of 2.

The procedure for measurement of receptor affinity is based on the ability of a compound to prevent specific binding of [^{3}H]-dihydromorphine (Terenius, 1974a). A fraction enriched in synaptic plasma membranes was obtained from rat brain from which the cerebellum had been removed. The standard incubation mixture contained 0.8 nM [^{3}H]-dihydromorphine and a receptor fraction (0.4 mg of protein) in 0.4 ml of a solution of the following composition (mM): NaCl 124, KCl 5, KH_2PO_4 1.2, $CaCl_2$ 0.75, $MgSO_4$ 1.3, N-2-hydroxy-ethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 26, the pH was adjusted to 7.4 with 1 M NaOH. Incubations, which were at 25°C for 40 min, were terminated by centrifugation. The radioactivity of the resulting pellets was measured by liquid scintillation spectrometry. In all experimental runs, samples containing an excess of unlabelled dihydromorphine (1 μM) were included; residual binding in these samples was considered to be nonspecific and subtracted from the experimental values (Goldstein, Lowney & Pál, 1971; Terenius, 1973; Pert & Snyder, 1973). The inhibitory effects of different concentrations of the test substances were expressed in % of the value obtained for specific binding of [^{3}H]-dihydromorphine. Each substance was tested at 4 or 5 concentrations in triplicate; values at each point did not alter normally by more than 10%. Each dose-response curve was repeated at least once.

Drugs

The drugs used were as follows: normorphine hydrochloride, (−)-phenazocine hydrobromide, NIH 8152 as the hydrochloride (Dr E.L. May), ketocyclazocine and ethylketocyclazocine as free bases (Sterling-Winthrop; Dr S. Archer), Mr 1268 and Mr 1353 as the methanesulphonates and Mr 2034 as free base (C.H. Boehringer Sohn, Ingelheim; Dr H. Merz). The structures are given in Table 1. Stock solutions of the salts were made in distilled water, those of the bases after the addition of the required amount of HCl. Concentrations are given in nM.

[^{3}H]-dihydromorphine (52 Ci/mmol) was obtained from the New England Nuclear Corporation (Boston, Mass.).

Results

Relative agonist potencies

The benzomorphans which were assayed had no antagonist activity in rodent antinociceptive tests (Archer & Pierson, personal communication, 1972; Merz *et al.*, 1974; Merz, Stockhaus & Wick, personal communication, 1975) or in the guinea-pig ileum and mouse vas deferens (Kosterlitz & Waterfield, 1975; Kosterlitz, Waterfield & Leslie, 1975; unpublished observations). The dose-response curves for their agonist actions were parallel to that of normorphine or morphine in both *in vitro* preparations.

There were four compounds which had no physical dependence capacity in the morphine-dependent monkey, and one compound which had not been tested on the monkey (Table 1). Two of

Table 1 Relative agonist potencies of benzomorphans in the guinea-pig ileum and the mouse vas deferens

Compound	*Structure*	*Physical dependence capacity**	*Relative agonist potency (normorphine = 1)*		*Mvd/Gpi*
			Guinea-pig ileum	*Mouse vas deferens*	
Normorphine	—	high	1	1	1
Phenazocine (NIH 7613)	(—)-α-5,9-dimethyl-2-phenethyl-2′-hydroxy-6,7-benzomorphan	high	13.3 ± 1.1 (4)	19.0 ± 3.6 (3)	1.4
NIH 8152	(±)-β-2,9-dimethyl-5-*n*-propyl-2′-hydroxy-6,7-benzomorphan	high	2.60 ± 0 29 (4)	4.17 ± 0.52 (4)	1.6
Ketocyclazocine (NIH 8847)	(—)-α-5,9-dimethyl-8-oxo-2-cyclopropylmethyl-2′-hydroxy-6,7-benzomorphan	none	68.5 ± 4.3 (9)	16.3 ± 1.1 (6)	0.24
Ethylketocyclazocine (NIH 8848)	(—)-α-9-methyl-5-ethyl-8-oxo-2-cyclopropylmethyl-2′-hydroxy-6,7-benzomorphan	none	397 ± 43 (7)	99 ± 11 (7)	0.25
Mr 1268 (NIH 8735)	(±)-α-5,9-dimethyl-2-(2-methyl-3-methylfuryl)-2′-hydroxy-6,7-benzomorphan	none	1.49 ± 0 15 (6)	0.34 ± 0.03 (6)	0.23
Mr 1353 (NIH 8737)	(±)-α-5,9-dimethyl-2-(3-methylfurfuryl—2′-hydroxy-6,7-benzomorphan	none	5.55 ± 0.50 (6)	1.54 ± 0.10 (6)	0.28
Mr 2034	(—)-α-(1R,5R,9R)-5,9-dimethyl-2-(L-tetrahydrofurfuryl)-2′-hydroxy-6,7-benzomorphan	?	94.4 ± 11.3 (5)	22.0 ± 1 9 (4)	0.23

The values are the means ± s.e. mean; the number of observations are given in parentheses. * Data obtained from Eddy & May (1966); Deneau, Villarreal & Seevers (1966); Villarreal & Seevers (1972); Swain & Seevers (1974).

them were ketocyclazocines, two were N-dimethylfuryl analogues of cyclazocine and one an N-tetrahydrofurfuryl analogue. Racemates were used or the (−)-isomers; the (+)-isomers were inactive (Kosterlitz, Waterfield & Berthoud, 1973; Kosterlitz *et al.*, 1975). Their relative agonist potencies, referred to normorphine as standard, varied between 1.5 and 400 in the guinea-pig ileum and 0.3 and 100 in the mouse vas deferens. The ratio, potency in mouse vas deferens/potency in guinea-pig ileum, varied only little around 0.25.

In contrast, the two benzomorphans with high physical dependence capacity, (−)-phenazocine and NIH 8152, had similar relative agonist potencies in the two preparations, the mouse vas deferens being somewhat more sensitive than the guinea-pig ileum. This good correlation is in agreement with the findings obtained on all other agonist compounds with either no or only negligible antagonist activity (Hughes *et al.*, 1975b).

Sensitivity to the antagonist effect of naloxone

The sensitivity of agonists to the antagonist action of naloxone is measured by the equilibrium dissociation constant, K_e. The values for seven agonists with relative potencies from 0.007 (codeine) to 690 (etorphine) varied between 1.2 and 4.0 nM, giving a mean of 2.2 nM (Kosterlitz, Lord & Watt, 1972).

Table 2 Potency of naloxone to antagonize the agonist effects of normorphine and the benzomorphans listed in Table 1

Compound	*Guinea-pig ileum*		*Mouse vas deferens*	
	Slope	K_e *(nM)*	*Slope*	K_e *(nM)*
Normorphine	0.95 ± 0.03	1.89 ± 0.15 (12)	0.98 ± 0.05	3.10 ± 0.25 (6)
Phenazocine	0.93 ± 0.06	2.08 ± 0.39 (5)	1.06 ± 0.01	4.05 ± 0.19 (3)
NIH 8152	0.84 ± 0.05	1.26 ± 0.31 (4)	—	—
Ketocyclazocine	1.04 ± 0.07	15.2 ± 3.1 (3)	—	—
Ethylketocyclazocine	1.04 ± 0.05	14.9 ± 0.9 (4)	0.98 ± 0.08	11.0 ± 0.6 (3)
Mr 1268	0.67 ± 0.02	—	—	—
Mr 1353	0.91 ± 0.06	11.7 ± 1.0 (4)	—	—
Mr 2034	1.08 ± 0.02	10.4 ± 1.8 (7)	1.00 ± 0.04	9.07 ± 0.71 (3)

The values are the means ± s.e. mean; the number of observations are given in parentheses. The values of K_e, the equilibrium dissociation constant, were calculated from the intersection with the abscissa of the regression line obtained by plotting log (dose-ratio −1) against log[naloxone]. When the slope had a value of less than 0.8, the antagonism was considered to be non-competitive (Mr 1268) and no K_e could be given. NIH 8152, the ketocyclazocines, Mr 1268 and 1353 were racemates, the remaining compounds the (−)-isomers.

Table 3 Correlation between affinity to the opiate receptors in rat brain and agonist activity in the guinea-pig ileum and mouse vas deferens

Compound	*Inhibition of [^{3}H]-dihydromorphine binding*	*Relative affinity (normorphine = 1)*	*Relative agonist potency/ relative affinity (normorphine = 1)*	
	ED$_{50}$ (nM)		*Guinea-pig ileum*	*Mouse vas deferens*
Normorphine	15	1.0	1.0	1.0
(−)-Phenazocine	0.8	19	0.7	1.0
(−)-Ketocyclazocine	2.0	7.5	9.1	2.2
(−)-Ethylketocyclazocine	0.35	43	9.2	2.3
Mr 1268	17	0.88	1.7	0.39
Mr 1353	10	1.5	3.7	1.0
Mr 2034	0.4	38	2.5	0.6

The K_e of naloxone determined for normorphine, (−)-phenazocine and NIH 8152 as agonists varied in the guinea-pig ileum between 1.3 and 2.1 nM (Table 2). In the less robust mouse vas deferens, such estimations were more difficult because of the long duration of the assay; the values for normorphine and phenazocine varied between 3 and 4 nM.

When the K_e values of naloxone were determined for the benzomorphans with no physical dependence capacity, they varied between 10 and 15 nM in the guinea-pig ileum and 9 and 11 nM in the mouse vas deferens. These results mean that, in the guinea-pig ileum, these benzomorphans require 6 to 7 times more naloxone for antagonism than normorphine. On the other hand, the two benzomorphans with high physical dependence capacity were as sensitive to the action of naloxone as was normorphine. Although there are fewer data for the mouse vas deferens, the relationship is similar, the benzomorphans with no physical dependence capacity requiring about 3 times more naloxone than normorphine.

Correlation between agonist potency and inhibition of specific [^{3}H]-dihydromorphine binding

The benzomorphans show specific affinities to the opiate receptors as demonstrated by their ability to inhibit specific binding of [^{3}H]-dihydromorphine to membrane fragments of rat brain (Table 3). The slopes of the dose-inhibition curves were not different from those obtained for morphine or other agonists (Terenius, 1974b). The rank order of their relative affinities is highly correlated to that of their relative agonist potencies in both *in vitro* preparations. When the ratios of relative agonist potency to relative affinity were calculated, their values were closer to the expected value of unity in the mouse vas deferens than in the guinea-pig ileum (Table 3).

Discussion

In their report on the effects of the N-cyclopropylmethyl - 5,9 - dialkyl - 2′ - hydroxy - 8 - oxo 6,7-benzomorphans, Swain & Seevers (1974) stressed the very unusual nature of these compounds. They neither suppressed nor precipitated abstinence in morphine-dependent monkeys. Similar findings had been obtained by Villarreal & Seevers (1972) on the N-dimethylfuryl-5,9-dimethyl-2′-hydroxy-6,7-benzomorphans. The importance of these findings lies in the fact that these substances are compounds which have potent antinociceptive effects without antagonist activity and yet have no physical dependence capacity in monkeys.

The results obtained in the guinea-pig ileum and mouse vas deferens underline their unusual pharmacological properties. The low sensitivity of the mouse vas deferens to the agonist action of these drugs and the lowered effectiveness of naloxone as an antagonist indicate that the mode of action of these drugs is probably different from that of agonists of the morphine type. It has been shown previously (Kosterlitz *et al.*, 1972) that the antagonist potency of naloxone is lower against the agonist actions of compounds with dual agonist and antagonist effects, such as nalorphine, levallorphan and diprenorphine, than against morphine. The benzomorphans investigated now, are the first compounds without antagonist component to show this phenomenon. Notwithstanding the close rank order correlation between the affinity of these compounds to the opiate receptors and their agonist potencies in both *in vitro* preparations, it has to be considered that they may act on a receptor different from that which mediates the effects of the agonists of the morphine type. In this connection it is of interest to note that the endogenous ligand enkephalin, which is an agonist without antagonist activity, also requires more naloxone for its antagonism (Hughes, Smith, Morgan & Fothergill, 1975a; Kosterlitz & Hughes, 1975).

The three assays described may thus be suitable for the selection of antinociceptive compounds that will not produce physical dependence, always providing that freedom from this property in monkeys also holds for man.

Supported by grants from the U.S. National Institute on Drug Abuse (DA 00662) and the U.S. Committee on Problems of Drug Dependence, NRC-NAS. F.M.L. is a Medical Research Council Research Student. L.T. was supported by the Swedish Medical Research Council. Grateful acknowledgement is made for the generous supply of the drugs mentioned in the Methods section.

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(Received July 14, 1975.)

Br. J. Pharmac. (1975), 55, 547-554

TOLERANCE TO SYMPATHOMIMETIC BRONCHODILATORS IN GUINEA-PIG ISOLATED LUNGS FOLLOWING CHRONIC ADMINISTRATION *in vivo*

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1 Pretreatment of the guinea-pig subcutaneously three times daily with either 5 μg/kg isoprenaline or adrenaline reduced the response of the isolated perfused histamine-constricted lung when challenged with either the same or a different sympathomimetic bronchodilator. The longer the animals were pretreated and the higher the dose of bronchodilator the greater was the degree of tolerance developed.

2 Tolerance was developed to aminophylline in the same preparation when the guinea-pig had been pretreated with aminophylline or isoprenaline. Cross-tolerance also developed to adrenaline when the guinea-pig had been pretreated with aminophylline.

3 Tolerance was still persistent in guinea-pigs one and two weeks after pretreatment three times daily with either isoprenaline or adrenaline (5 μg/kg s.c.) for seven days in the same preparation. Only after three weeks was the tolerance diminished.

4 These results suggest that asthmatic patients who use bronchodilators excessively may become refractory to the bronchodilator effect of these drugs. They also support the hypothesis that induced cross-resistance to endogenous sympathetic stimulation could lead to a deterioration of the asthmatic state in patients using the sympathomimetic bronchodilators and that this may explain the increase in asthma mortality rate.

5 A mechanism of tolerance to sympathomimetic bronchodilators is postulated.

Introduction

The asthma mortality rate in England and Wales increased during the period 1959-1966. This increase was real and not caused by environmental hazards or the wrong diagnosis of asthma death. It was related to the use of pressurized aerosols containing sympathomimetic bronchodilators which had become popular in the treatment of asthma since 1960 (Speizer, Doll & Heaf, 1968; Speizer, Doll, Heaf & Strang, 1968). Since 1968 further reports have been published which show a positive correlation between the use of pressurized aerosols containing sympathomimetic bronchodilators and an increase in the asthma mortality rate (Inman & Adelstein, 1969; Fraser, Speizer, Waters, Doll & Mann, 1971; Stolley, 1972).

Attempts have been made to find how these pressurized aerosols may cause the observed increase in asthma death. One explanation suggested by Conolly, Davies, Dollery & George (1971) is that excessive use of sympathomimetic amines by patients produces a state of drug-induced cross-resistance to endogenous sympathetic stimulation in the respiratory tract, which might lead to a deterioration in asthmatic state. The authors suggested that this might explain the rise in asthma mortality rate. The mortality experiments of Conolly *et al.* (1971) were verified by Bouhuys, Douglas & Lewis (1972).

In this paper a simple method has been used to investigate the development of tolerance and cross-tolerance to the bronchodilator effect of sympathomimetic amines in guinea-pigs. Recovery from this tolerance is also described.

Methods

The method was a modified version of that described by Sollman and von Oettingen (1928) and Tainter, Pedden & James (1934).

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A guinea-pig of either sex and weighing 400-500 g was killed by a blow on the head. The trachea and lungs were dissected free, and the trachea tied to a plastic cannula which was connected to a three-way tap through which drug solutions were injected. Slight scarifications were made along each lobe of the lungs with a fine needle. The trachea and lungs suspended from the cannula were surrounded by a heating jacket which was connected to a circulating water bath, so that the lungs were maintained at a temperature of 36-37° C. The lungs were perfused with Krebs solution of the following composition (g/litre distilled water):– NaCl 6.9, KCl 0.35, $CaCl_2$ 0.28, $MgCl_2$ 1.1, $NaHCO_3$ 2.1, NaH_2PO_4, $2H_2O$ 0.182, glucose 2. This solution was constantly aerated with a mixture of 95% O_2 and 5% CO_2 and passed through a coil immersed in a temperature-regulated water bath, so that the solution reached the cannula inserted into the lungs at 36-37° C. The Krebs solution then passed down the trachea through the bronchi, escaped from the alveoli through scratches on the surface of the lungs and was collected in a funnel. It then passed through a silicon rubber tube and entered a float recorder, which was a modified version of that described by Bülbring, Crema & Saxby (1958). When the Krebs solution reached a predetermined level in the measuring chamber of the flow recorder it emptied automatically. This caused a deflection to be recorded on smoked paper. Bronchoconstriction resulted in a reduced rate of flow and fewer signals on the smoked paper in a given time interval, whereas bronchodilation increased the rate of flow and frequency of signals.

Histamine was used as a bronchoconstrictor. The maximum response to histamine was obtained with a dose of 500 μg, and the response remained at this level for at least half an hour. Histamine was injected first, and the response recorded for 15 minutes. This was followed by injection of the bronchodilator drug, and the response to this drug recorded for 15 minutes. Doses of histamine and bronchodilator drugs were alternated in this way until the tissue failed to respond to the drugs. Tachyphylaxis to histamine developed in the isolated lungs. In order to compensate for this, the different doses of the bronchodilator drug given to the isolated lungs in all experiments were randomized.

Injection of solutions at room temperature into the isolated lungs was found to increase the flow rate of Krebs solution, so all injections of drugs were followed by injections of Krebs solution; thus a constant volume (1 ml) was given on each occasion. Control determinations, where the animals were not pretreated with bronchodilator drugs, were repeated at regular intervals throughout the experimental period. Each control curve shown in the results was the mean of a minimum of 15 determinations.

The animals were pretreated with bronchodilator drugs administered subcutaneously. Each dose of bronchodilator was given in 0.5 ml solution per kg body weight, three times daily for a varying number of days (1-14 days). Doses were calculated to body weight and chosen to be approximately the dose which an asthmatic patient might use in an attack. After the last injection, the animals were left overnight and killed the following morning. The number of animals used for each log dose-response curve was at least four, but usually more.

Drugs that were investigated were adrenaline acid tartrate, isoprenaline sulphate, salbutamol sulphate, terbutaline sulphate, aminophylline and histamine acid phosphate. The solutions of adrenaline, isoprenaline, salbutamol and terbutaline were stabilized with ascorbic acid 200 μg/ml.

To investigate the recovery from tolerance, the animals were killed 7, 14 and 21 days after completion of the course of pretreatment with isoprenaline or adrenaline for 3 or 7 days.

Increases in basal flow rates caused by increasing doses of bronchodilators were found to follow a log dose-response relationship. Tolerance to a bronchodilator was recognized as a downward displacement of the log dose-response curve of the isolated perfused histamine-constricted lungs of guinea-pigs which had been pretreated with either the same or a different bronchodilator when compared with the control curve of non-pretreated animals.

Statistical analysis was applied to the results obtained to test whether the log dose-response curves were altered. The significance of the vertical distance between the response mean of each curve was tested (Moore & Edwards, 1965). This parameter was selected in preference to the more conventional 'shift to the right' because in so many cases it proved impracticable to determine complete dose-response curves for each experiment.

Results

There was a downward displacement of the log dose-response curve of the isolated histamine-constricted lungs of the guinea-pig to adrenaline when the animals were pretreated for three days with adrenaline 5 μg/kg subcutaneously thrice daily ($P < 0.01$) (Figure 1).

The downward displacement of the curve was increased when the animals were pretreated for a longer period of time. Following pretreatment for

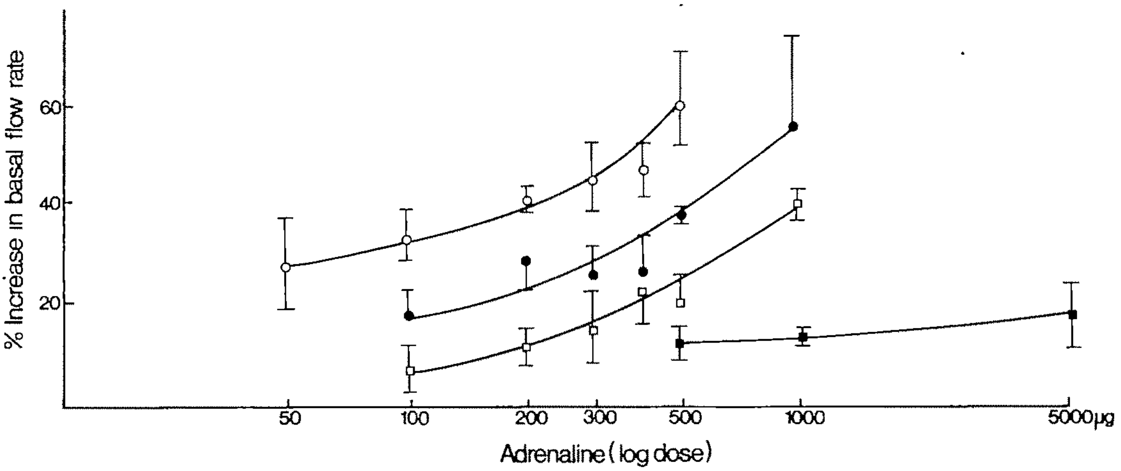

Figure 1 The effect of pretreatment with adrenaline (5 µg/kg) on the response of isolated perfused histamine-constricted guinea-pig lungs to adrenaline. (○) Control; (●) 3 days pretreatment, (□) 7 days pretreatment; (■) 14 days pretreatment. Vertical lines show s.e. mean.

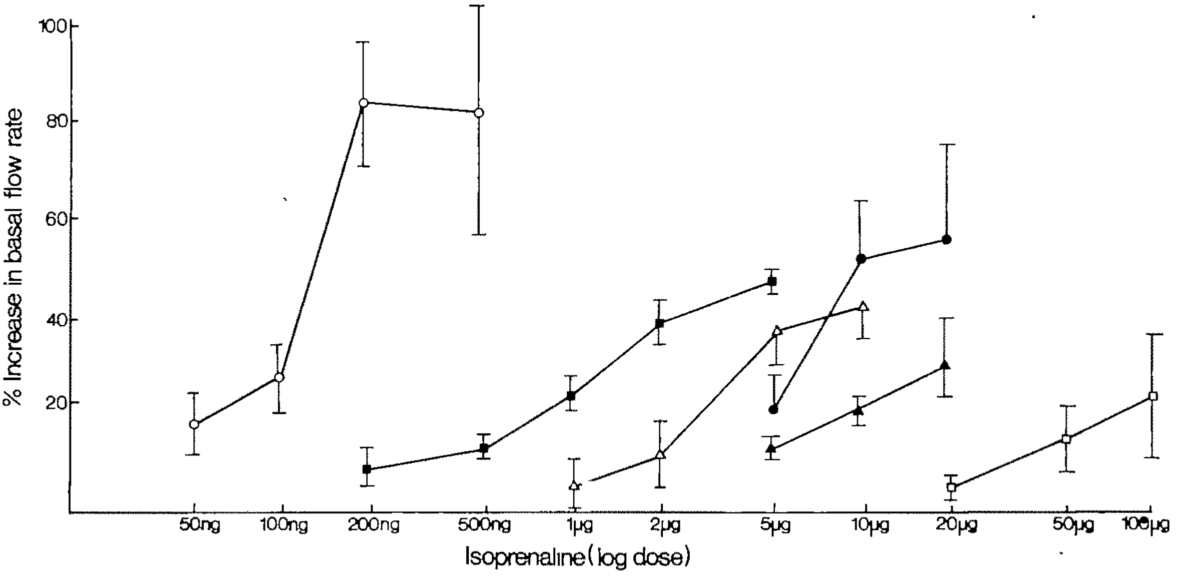

Figure 2 The effect of pretreatment with isoprenaline (5 µg/kg) or adrenaline (5 µg/kg) on the response of the isolated perfused histamine-constricted guinea-pig lung to isoprenaline. (○) Control; (■) 1 day pretreatment with isoprenaline; (△) 2 days pretreatment with isoprenaline, (●) 4 days pretreatment with isoprenaline; (□) 7 days pretreatment with isoprenaline, (▲) 4 days pretreatment with adrenaline. Vertical lines show s.e. mean.

two weeks, no significant response was obtained to doses of up to 5 mg.

When the animals were pretreated with a higher dose (10 µg/kg), the downward displacement of the log dose-response curve was increased.

Pretreatment of the animals with ascorbic acid 200 µg/ml in 0.9% w/v NaCl solution resulted in a response of the isolated histamine-constricted lungs to adrenaline which was not significantly different from that of non-pretreated animals.

A similar downward displacement of the log dose-response curves to isoprenaline was observed when the animals were pretreated with isoprenaline 5 µg/kg subcutaneously thrice daily for

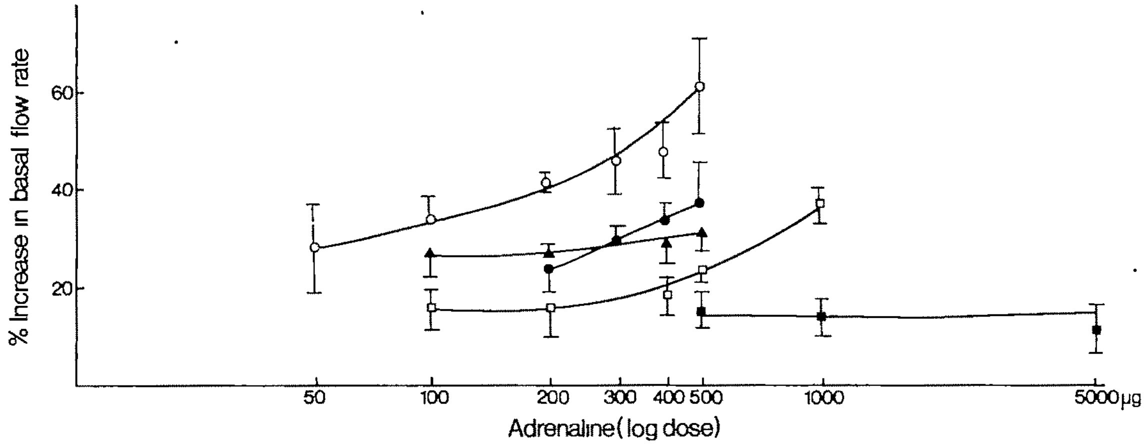

Figure 3 The effect of pretreatment with isoprenaline (5 μg/kg) on the response of isolated perfused histamine-constricted guinea-pig lungs to adrenaline. (○) Control; (●) 3 days pretreatment; (▲) 4 days pretreatment; (□) 7 days pretreatment; (■) 14 days pretreatment. Vertical lines show s.e. mean.

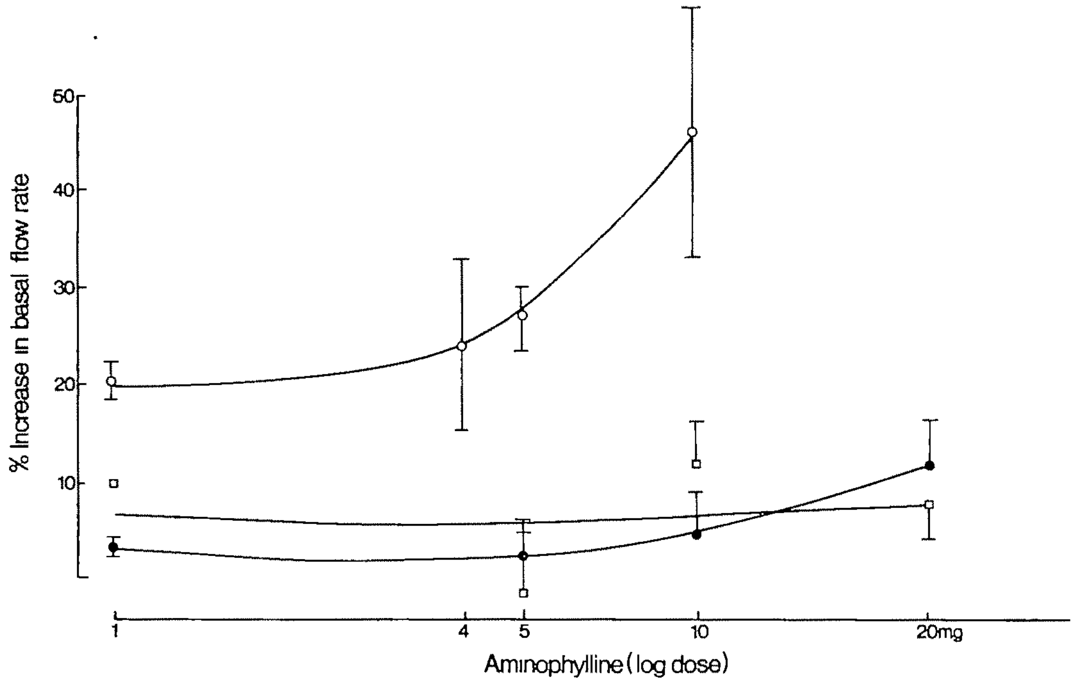

Figure 4 The effect of pretreatment with aminophylline (5 mg/kg) or isoprenaline (5 μg/kg) on the response of isolated perfused histamine-constricted guinea-pig lungs to aminophylline. (○) Control; (●) pretreated with aminophylline for 7 days; (□) pretreated with isoprenaline for 7 days. Vertical lines show s.e. mean.

one day ($P < 0.05$) (Figure 2). Increased pretreatment times produced further marked displacement of the curves to the right. The displacement of the curve was even more marked with an increased pretreatment dose (15 μg/kg). A similar effect was observed when the animals were pretreated with adrenaline and challenged with isoprenaline (Figure 2) or pretreated with iso-

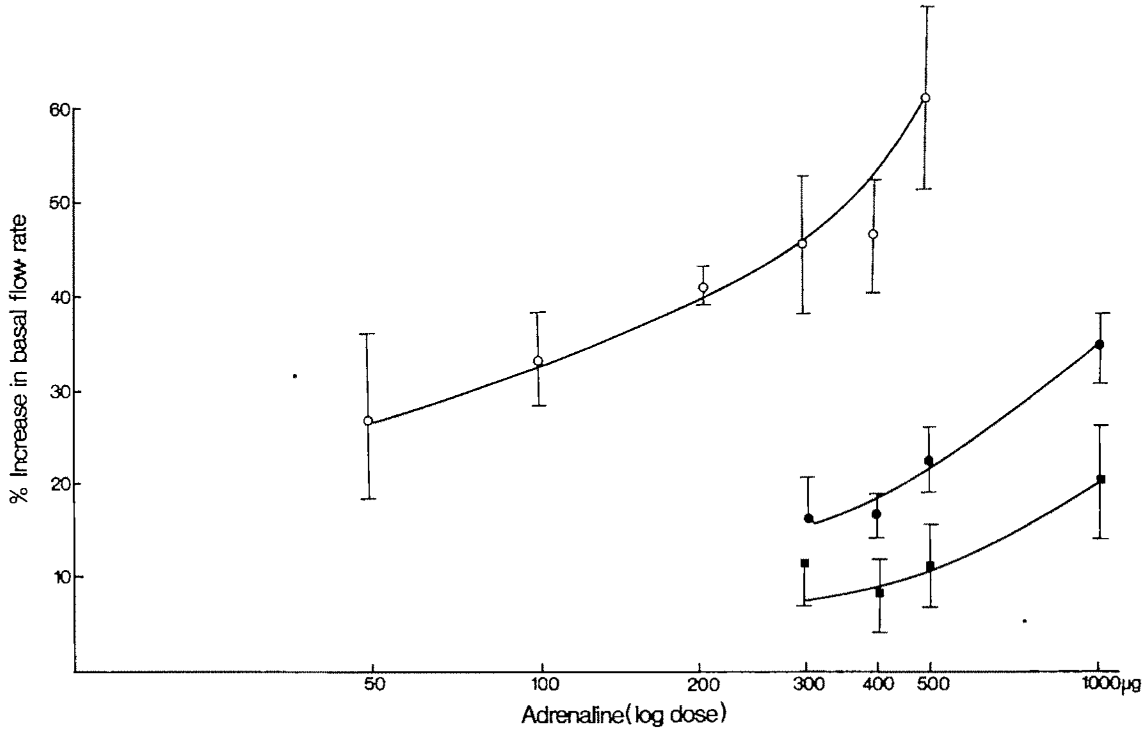

Figure 5 The effect of pretreatment with terbutaline (5 µg/kg) or aminophylline (5 mg/kg) on the response of isolated perfused histamine-constricted guinea-pig lungs to adrenaline. (o) Control; (•) pretreated with terbutaline for 3 days; (▪) pretreated with aminophylline for 7 days. Vertical lines show s.e. mean.

prenaline and challenged with adrenaline (Figure 3) or pretreated with salbutamol and challenged with salbutamol.

When the animals were pretreated with aminophylline 5 mg/kg or isoprenaline 5 µg/kg subcutaneously thrice daily for seven days, the log dose-response curve to aminophylline was displaced downwards ($P < 0.01$) (Figure 4). A dose of up to 25 mg did not produce a significant response in the isolated lung preparation.

Similarly, the log dose-response curve to adrenaline was displaced downwards when the animals were pretreated with terbutaline 5 µg/kg thrice daily for three days ($P < 0.001$) or aminophylline 5 mg/kg thrice daily for seven days ($P < 0.001$) (Figure 5).

When the guinea-pigs were left for three weeks after seven days of isoprenaline pretreatment the log dose-response curves of adrenaline in these animals were significantly displaced upwards ($P < 0.01$) compared with the curves of animals left for one week and two weeks after seven days of isoprenaline pretreatment (Figure 6). Recovery from tolerance had begun to develop but was not yet complete.

Discussion

Tolerance Studies

Tolerance to the bronchodilator effect of adrenaline, isoprenaline, salbutamol and aminophylline developed in the isolated perfused histamine-constricted lungs of guinea-pigs which had been pretreated with adrenaline, isoprenaline, salbutamol and aminophylline. The degree of tolerance was dependent upon the amount and the number of doses given during the pretreatment stage. The longer the animals were pretreated and the higher the dose of bronchodilator given (isoprenaline or adrenaline) the greater was the degree of tolerance developed in the isolated lungs. Tolerance could develop to such a degree that regardless of the dose of bronchodilator used to challenge the lungs (adrenaline, isoprenaline or

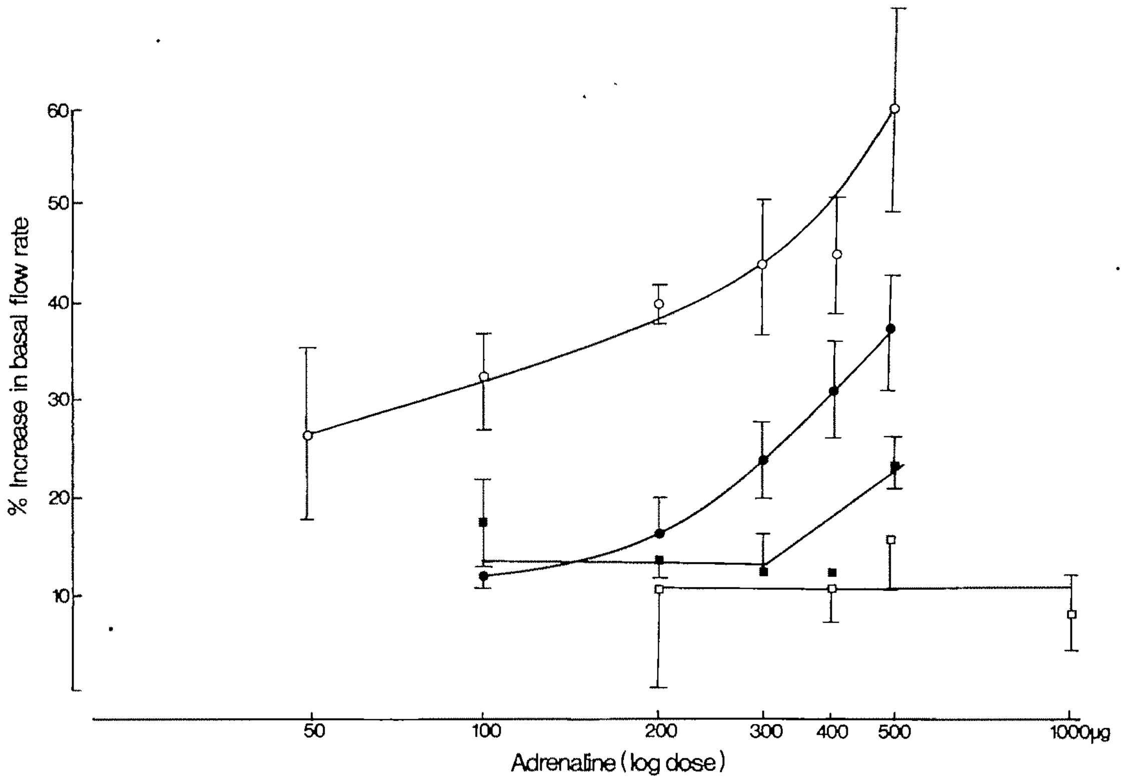

Figure 6 The recovery of the response of isolated perfused histamine-constricted guinea-pig lungs to adrenaline after pretreatment with isoprenaline (5 µg/kg) thrice daily for seven days. (○) Control; (□) animals left for 1 week; (■) animals left for 2 weeks; (●) animals left for 3 weeks Vertical lines show s.e. mean.

aminophylline) no significant bronchodilation was produced.

Benson & Perlman (1948) and Laurence & Moulton (1960) reported that tolerance developed to the bronchodilator effect of adrenaline after excessive use of this drug in man. Fleish & Titus (1972) showed that tachyphylaxis to the vasodilator effect of isoprenaline developed in the rat aorta and Paterson, Conolly, Davies & Dollery (1968) noticed that tolerance developed to the chronotropic effect of isoprenaline in asthmatic patients taking heavy doses of isoprenaline. Bouhuys *et al.* (1972) also observed the development of tachyphylaxis to isoprenaline in the guinea-pig isolated trachea.

These results suggest that asthmatic patients who are heavy users of bronchodilators may become resistant to these drugs. The value of these drugs in relieving mild asthmatic attacks in such patients becomes less. In an extreme case, a patient may die because the drug he is using has become completely ineffective. A young asthmatic has been found dead clutching an empty bronchodilator aerosol container (Pickvance, 1967). The same conclusion was reached by Herxheimer (1973).

Cross-tolerance studies

Cross-tolerance developed to the bronchodilator effect of adrenaline in the isolated histamine-constricted lung of the guinea-pig when the animals had been pretreated with isoprenaline, terbutaline or aminophylline. Cross-tolerance developed to isoprenaline when the guinea-pig was pretreated with adrenaline and also to aminophylline when the animals were pretreated with isoprenaline. The aminophylline experiments may not indicate whether tolerance is specific and/or non-specific, since aminophylline can be regarded as an indirectly acting sympathomimetic amine because it causes an increase in the release of endogenous catecholamines into the blood circulation (Atuck, Blaydes, Westervelt & Wood, 1967; Westfall & Fleming, 1968; Peach, 1972; Wooten, Thoa, Koplin & Axelrod, 1973). Sympathomimetic amines may also act in a way similar to aminophylline, since adrenaline inhibits phosphodiesterase activity (Hitchcock, 1973).

Conolly *et al.* (1971) reported that in guinea-pigs, the mortality from histamine-induced bronchospasm was higher in groups which had been pretreated with sympathomimetic amines than in groups which had been pretreated with saline. They suggested that in asthmatic patients who use sympathomimetic pressurized aerosols excessively cross-resistance develops to both exogenous sympathomimetic amines and to the natural transmitter release by the adrenergic nerves, and that this may lead to a deterioration of the asthmatic state and explain the rise in the asthma mortality rate. The cross-tolerance experiments decribed here may explain the good results which were obtained only after the withdrawal of adrenergic bronchodilator therapy from some asthmatic patients (Keighley, 1966, Reisman, Friedman & Arbesman, 1968).

Recovery experiments

The work described in this paper has demonstrated that the tolerance which developed as a result of the repeated administration of isoprenaline or adrenaline remained for periods of up to three weeks after the last administration of isoprenaline or adrenaline to guinea-pigs.

Mechanism of the development of tolerance

It has been suggested (Robison, Butcher & Sutherland, 1967; Sutherland, Robison & Butcher, 1968; Robison, Butcher & Sutherland, 1971) that the β-adrenoceptor is a component of the enzyme adenyl cyclase and that catecholamines stimulate adenyl cyclase to increase the formation of intracellular cyclic 3′,5′-adenosine monophosphate (cyclic AMP) from adenosine triphosphate (ATP) and that this causes relaxation of the smooth muscle of the bronchi.

Some years ago, Szentivanyi (1968) proposed that asthmatics had defective β-adrenoceptors. Amongst the evidence which supports this hypothesis are the following points: (1) The increase in urinary cyclic AMP in asthmatic patients following adrenaline administration was less than that of normal subjects (Fireman, 1973). (2) The increase in adenyl cyclase activity of leucocytes by isoprenaline from asthmatic patients is less than that of normal subjects (Logsdon, Middleton & Coffey, 1972). (3) Metabolic and circulatory responses to sympathomimetic amines were less in asthmatic patients than in normal subjects (Cookson & Reed, 1963; Lokey, Glennon & Reed, 1967; Middleton, Finke & Arce, 1968, Morris, 1971; Fireman, 1973).

The patients in whom these observations were made were asthmatic and had been using sympathomimetic bronchodilators until a few hours or a few days prior to these studies (Cookson & Reed, 1963; Lokey *et al.*, 1967; Middleton *et al.*, 1968). The resistance of these patients to these drugs might have been due not to an abnormality of the β-adrenoceptors, but to tolerance which may result from receptor desensitization, produced by the sympathomimetic amines. The idea is supported by the recent work of Nelson (1973) who found that in normal subjects after ephedrine administration, the metabolic and circulatory responses to adrenaline were less than in those who had not received ephedrine. It is also supported by the work of Franklin & Foster (1973) who reported that after pre-incubation of human diploid fibroblasts with isoprenaline for 2-2.5 h, subsequent challenge for 10 min with isoprenaline failed to induce any increase in cyclic AMP. They also suggested that desensitization to isoprenaline in these cells resulted from isoprenaline-induced conformational changes in the receptor which led to their inactivation.

The tolerance to aminophylline which occurred after a guinea-pig had been pretreated with isoprenaline may also support the idea that tolerance to isoprenaline is due to the effect of isoprenaline on adenyl cyclase. It is thought that aminophylline inhibits the phosphodiesterase which breaks down cyclic AMP and that the resulting increased concentration of cyclic AMP causes bronchial relaxation (Robison *et al.*, 1971).

However, if isoprenaline administration had altered the sensitivity of the β-adrenoceptors, and had reduced the activity of adenyl cyclase, this would explain why aminophylline was ineffective, because despite inhibition of phosphodiesterase there would be no increase of cyclic AMP and no muscle relaxation.

The authors wish to thank Miss D. Brown for her technical assistance, Mrs J. Babs for her criticism, Mr T. Soraham and Dr J. Waterhourse for statistical treatment of the results and Messrs Astra Chemicals for the supply of terbutaline sulphate.

The work was supported by a grant from the University of Benghazi, Libya.

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(Received August 21, 1975.)

THE ACTIONS OF FLUPENTHIXOL UPON 5-HYDROXYTRYPTAMINE-INDUCED AGGREGATION AND THE UPTAKE OF 5-HYDROXYTRYPTAMINE AND DOPAMINE BY HUMAN BLOOD PLATELETS

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The effects of the α- and β-isomers of flupenthixol on 5-hydroxytryptamine (5-HT)-induced platelet aggregation and on 5-HT and dopamine uptake were investigated. α-Flupenthixol was 185 times more potent than the β-isomer as an inhibitor of platelet aggregation. In contrast both isomers were equipotent as inhibitors of uptake of 5-HT and dopamine. The data suggest that 5-HT-induced aggregation and uptake are separate processes.

Introduction We are currently attempting to relate changes in the therapeutic efficacy of neuroleptic drugs used for the treatment of psychosis to changes in blood platelet aggregation responses. The basis for these experiments is that while Mills & Roberts (1967) and Boullin, Grahame-Smith, Grimes & Woods (1975a) found that chlorpromazine *in vitro* inhibited platelet aggregation responses to 5-hydroxytryptamine (5-HT), we subsequently observed enhanced aggregation responses to 5-HT in schizophrenics chronically treated with chlorpromazine (Boullin, Woods, Grimes, Grahame-Smith, Wiles, Gelder & Kolakowska, 1975b).

As the thioxanthine derivative flupenthixol, which is structurally related to the phenothiazines, is currently in use for the treatment of psychosis (Reiter, 1969), we thought it would be of interest to study the aggregation responses of patients treated with flupenthixol. However, recent work suggested to us that some preliminary experiments upon the effects of flupenthixol isomers *in vitro* should be made.

Flupenthixol shows geometrical isomerism existing as *cis* (α) and *trans* (β) forms. The α-form shows the greatest biological activity in the following experimental situations relating to central dopaminergic mechanisms: inhibition of dopamine-induced stimulation of adenylate cyclase of rat striatum (Miller, Horn & Iversen, 1974); inhibition of turning behaviour in rats induced by methamphetamine or apomorphine following the production of unilateral lesions of the substantia nigra with 6-hydroxydopamine (Kelly & Miller, 1975).

Additionally the α-isomer of flupenthixol is more potent than the β-isomer in a large number of behavioural tests in various species (Møller, Nielsen, Pedersen, Nymark, Franck, Boeck, Fjalland & Christensen, 1973).

In view of these results and the possible involvement of central dopaminergic mechanisms we have studied the effects of α- and β-flupenthixol upon platelet aggregation and upon 5-HT and dopamine uptake by platelets.

Methods Citrated platelet rich plasma (PRP) was prepared as described by Boullin, Green & Price (1972). 5-HT-induced aggregation was measured as described in previous papers (Boullin *et al.*, 1975a; Boullin *et al.*, 1975b).

The α- and β-isomers of flupenthixol were added to 1 ml samples of PRP at 37° C, 3 min before addition of 20 μM 5-HT to induce aggregation. The inhibitory potency of flupenthixol isomers was expressed as percentage inhibition determined by the total change in optical density of PRP produced by 5-HT in the presence of various concentrations of flupenthixol compared with the aggregation responses produced by 5-HT alone (Boullin *et al.*, 1975a).

5-HT and dopamine uptake into platelets was measured as described previously by Boullin & O'Brien (1970) using concentrations of 0.1 to 10 nmol/ml.

Results Both α- and β-isomers of flupenthixol inhibited 5-HT-induced aggregation and also 5-HT and dopamine uptake (Figure 1). However, there were differences in the inhibitory potencies of the two isomers in regard to these parameters. For example the ED_{50} for inhibition of 5-HT-induced aggregation was 0.07 nmol/ml for α-flupenthixol and 13.0 nmol/ml for the β-isomer. On the other hand, there was no difference in potency between the isomers in regard to inhibition of 5-HT or dopamine uptake. The ED_{50} for inhibition of 5-HT

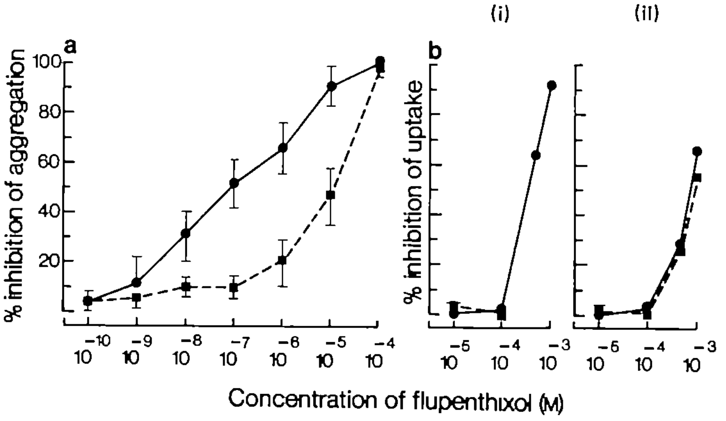

Figure 1 (a) Inhibition of platelet aggregation by α-flupenthixol (●), and β-flupenthixol (■) Aggregation was induced by 20 nmol/ml 5-hydroxytryptamine (5-HT). The values shown are the mean of six experiments. Vertical lines show s.e. mean. (b) Inhibition of the uptake of 10^{-6} M 5-HT (i) and 10^{-6} M dopamine (ii) by α- (●) and β (■) flupenthixol. The inhibition of 5-HT uptake by 10^{-4} M and 10^{-3} M flupenthixol was identical for both isomers and only one line is drawn (i). Each value is the mean of two determinations. Similar results were obtained using substrate concentrations of 10^{-5} M and 10^{-7} M. Note that there is a difference in potency between the two isomers only in regard to inhibition of aggregation.

uptake was 50 nmol/ml and 90 nmol/ml for dopamine uptake.

Discussion The data show that flupenthixol is an inhibitor *in vitro* of 5-HT-induced platelet aggregation. The ID_{50} for inhibition by the α-isomer is 0.07 nmol/ml which is comparable to the potency of the most active chlorpromazine metabolite (NOR_1 chlorpromazine) investigated in our earlier experiments (Boullin *et al.*, 1975a). Also of interest in regard to our clinical investigations (Boullin *et al.*, 1975b) is the fact that we have observed enhanced aggregation responses to 5-HT in a small number of patients receiving αβ-flupenthixol which is the form of the drug in therapeutic use (Present authors' unpublished observations). Thus there seems little doubt that flupenthixol is a suitable compound for investigating changes in the relationship between platelet aggregation responses and clinical effect in patients suffering from psychoses.

Regarding the pharmacological activity of flupenthixol isomers, our results confirm earlier reports (cited in the introductory section) that α-flupenthixol is more potent than the β-isomer in various pharmacological test systems.

It is of interest that the difference in potency between the two isomers of flupenthixol on platelet responses only applies to the physiological response of the cells to 5-HT, namely aggregation, and not to changes in 5-HT or dopamine uptake. These differences support the view that amine uptake and aggregation processes are dissociated in platelets. Bygdeman & Johnson (1971) first reported a dissociation between noradrenaline-induced aggregation and uptake, while Born, Juengjaroen & Michal (1972) reached a similar conclusion with 5-HT. Our results agree with the viewpoint of the earlier workers, suggesting that the mechanisms involved in 5-HT-induced platelet aggregation are quite different from those involved in 5-HT uptake. Furthermore, our results indicate that the differences in potency between the two isomers of flupenthixol are not confined to dopaminergic systems (Kelly & Miller, 1975) but also extend to the 5-HT-mediated aggregation response.

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(Received August 11, 1975.)

Br. J. Pharmac. (1975), 55, 558-560

POSTNATAL DEVELOPMENT OF DOPAMINE DEAMINATION IN THE STRIATUM OF THE RAT

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In the striata of litter-mate rats the development of the monoamine oxidase (MAO) activity towards dopamine *in vitro* followed a similar time course with age as the tissue concentrations of homovanillic acid and dihydroxyphenylacetic acid, the two acid metabolites of dopamine formed by the action of MAO.

Introduction During a study of the development of the enzyme monoamine oxidase (E.C. 1.4.3.4.; MAO) it has been observed that the ability of homogenates of the striata of 5 and 10 day old rats to deaminate dopamine was very low or not detectable. Depending on the strain of rats, this was followed by a more or less steep rise in dopamine deamination and a maximum was reached around days 30 – 40. In contrast to dopamine, considerable quantities of tyramine were metabolized by the same striatal homogenates of the 5 day old rats. Deamination of tyramine also increased up to day 40, but the rate of increase with age was slower than that for dopamine (Blatchford, Holzbauer & Youdim, 1975; Youdim & Holzbauer, 1975).

Our observations were made on tissue homogenates *in vitro*. The question arose whether the *in vitro* observations reflected the rates at which dopamine is being deaminated *in vivo*. As an index of the *in vivo* deamination the concentrations in the striatum of the two naturally occurring acidic metabolites of dopamine, homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC) can be used.

That dopamine deamination in the striatum is low in the young animal has been indicated in previous experiments. Thus in the sheep the concentration of HVA in the caudate nucleus was found to increase between foetal and adult life (Sharman, 1963). Keller, Bartholini & Pletscher (1973) have measured striatal concentrations of dopamine and HVA in Wistar rats of the Füllinsdorf colony aged between 4 and 60 days. In these rats only small quantities of HVA were detected at day 4. The value obtained on day 12 was no longer significantly different from the highest value, which was found on day 18. Because of the strain differences observed in our previous experiments a direct comparison between these *in vivo* studies and our *in vitro* studies was not possible. In the present work we have, therefore, used litter-mate rats to compare directly the development of MAO activity towards dopamine in the striatum *in vitro*, with the striatal concentrations of both acidic dopamine metabolites, HVA and DOPAC.

Methods Rats from 14 litters born between March 9th and 26th 1975 of mothers belonging to the same Wistar colony were used. The tissues from these rats were distributed between the samples to be analysed for enzyme activity and phenolic acid so that each age group included the striata from at least one rat from each litter. Each sample contained either male or female tissue. No significant sex differences were seen in the phenolic acid concentration or MAO activity. The rats were weaned at day 21. They were killed by rapid decapitation. MAO activity was measured in the striata from one rat. For the measurement of HVA and DOPAC at days 5, 8 and 10 the striata of 5 rats were pooled for each sample. At day 20, three rats were used per sample and, at day 30, two rats. The acids were also measured in individual striata of 6 of the mother rats.

For monoamine oxidase assay the tissues (which were kept up to 14 days at −18° C between dissection and assay) were homogenized in 1 ml 0.1 M sodium phosphate buffer pH 7.4. MAO activity was assayed by a radiochemical method similar to that of Robinson, Lovenberg, Keiser & Sjoerdsma (1968). Each assay sample consisted of 0.4 ml buffer to which 0.1 ml tissue homogenate was added and, after a preincubation period of 5 min at 37° C, 0.1 ml of substrate solution. The substrate solution contained 100 nmol of cold dopamine or tyramine per 0.1 ml plus approx. 50,000 d/min of ^{14}C-labelled amine. The samples were incubated for 30 min at 37° C and then passed through a column (approx. 0.5 x 2.5 cm) of Amberlite CG50 (mesh 200-400) to separate the amines, which were retained on the column, from

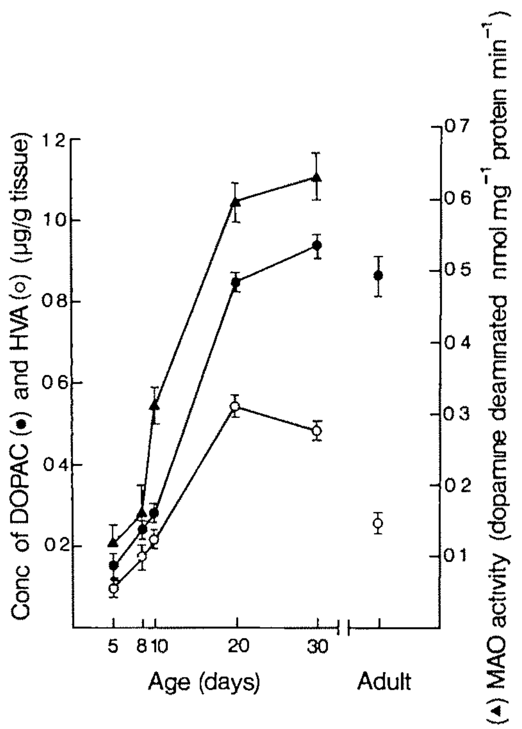

Figure 1 Postnatal development of dopamine deamination in rat striatum. Mean values are given; vertical lines show s.e. mean. (▲) Monoamine oxidase (MAO) activity towards dopamine; (●) dihydroxyphenylacetic acid (DOPAC); (○) homovanillic acid (HVA). Number of samples for each mean value: MAO: day 5: 12; day 8: 5; day 10: 12; day 20: 7; day 30: 10. HVA and DOPAC: day 5: 8; day 8. 4; day 10: 7; day 20: 8; day 30: 8; adults: 6.

their metabolites. The radioactivity of the metabolites was counted in a liquid scintillation spectrometer using Unisolve scintillation fluid. The protein content of the tissue homogenates was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951). The results were expressed in nmol amine deaminated/mg protein per minute.

For the assay of HVA and DOPAC the striatial tissue immediately after removal from the brain was frozen on a sheet of aluminium foil placed over liquid N_2 and then stored for up to 10 days, wrapped in aluminium foil, immersed in liquid N_2. The phenolic acids were estimated by a fluorimetric method based on that of Murphy, Robinson & Sharman (1969). The mean recovery for DOPAC was 54% and for HVA 69%. All values were corrected for losses.

Results Figure 1 shows the simultaneous rise in the concentrations of HVA and DOPAC in the rat striatum with increasing age and the ability of the tissue homogenates to deaminate dopamine *in vitro*. The estimates for the phenolic acids and for the MAO activity obtained on day 5 were at the lower limits of the estimation methods. The increase in MAO activity towards dopamine which occurred with age were similar to the increases in the concentrations of HVA and DOPAC in the striatum. Between days 8 and 20 dopamine deamination increased 3.8 fold; during the same period the rise in the striatal concentration of HVA was 3.2 fold and that of DOPAC 3.5 fold. The ratio of DOPAC to HVA lay between 1.3 and 1.7 until day 20 when the HVA concentrations reached a maximum. On day 30 the DOPAC concentration was about 10% higher than on day 20 and the ratio DOPAC to HVA was 1.9. In the adult rats the concentrations of both acids were lower than in their 30 day old offspring and the ratio DOPAC/HVA was 3.4.

The rise in MAO activity towards tyramine with age was slower than that towards dopamine. On day 5, four times more tyramine than dopamine was deaminated by the same homogenates whereas on day 30 only twice as much tyramine as dopamine was deaminated. There was a twofold rise in the deamination of 5-hydroxytryptamine (5-HT) between the 5 and 10 day old rats. At both ages the rates of deamination of 5-HT by striatal homogenates were similar to those of tyramine.

Discussion When measuring *in vitro* activities of enzymes in tissue homogenates the question arises whether such measurements can be used as an indication for the events occurring *in vivo*. From the present findings it appears that the increase in the MAO activity towards dopamine during the first 4 weeks of life parallels the development of the *in vivo* deamination of dopamine in the striatum of the rat. The validity of this interpretation depends on the premise that the disposition of the phenolic acids does not change with age.

The development of the striatal catabolism of dopamine closely follows the anatomical development of the dopaminergic neurones. In a detailed fluorescence-histochemical study Loizou (1972) showed that during the first week after birth the presumed dopamine containing varicosities are sparse. The rate of development thereafter was strain dependent. However, after the third week the varicosities became individually non-distinct as in the adult rat, indicating the proliferation of axon terminals. Thus the *in vivo* and *in vitro* deamination of dopamine seems closely related to the development of the dopamine containing neurones. However, this may be a purely fortuitous occurrence as the development of dopamine deamination *in vitro* follows a similar time course with age in other brain regions (e.g. the cerebellum) in which no extensive dopamine containing systems have so far been described. This is also true for the adrenal glands and the heart, but not for the liver (Youdim & Holzbauer, 1975).

In confirmation of previous findings (Blatchford *et al.*, 1975; Youdim & Holzbauer, 1975), there was a difference in the increase with age between the rates at which dopamine and tyramine were deaminated by homogenates of rat brain striata.

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(Received September 4, 1975.)

Br. J. Pharmac. (1975), 55, 561-562

γ-AMINOBUTYRIC ACID RECEPTOR ON VASCULAR SMOOTH MUSCLE OF DOG CEREBRAL ARTERIES

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γ-Aminobutyric acid (GABA) had a relaxant effect on dog cerebral arteries which was blocked only by picrotoxin, a known antagonist of GABA receptors. Other agents, adrenoceptor and ganglion blocking agents, atropine, reserpine, tetrodotoxin and ouabain, had no effect on the inhibitory action suggesting the existence of GABA receptors in the vascular smooth muscle of dog cerebral arteries.

Introduction In spite of overwhelming interest in the mechanisms influencing the control of cerebral blood circulation, comparatively few studies have been concerned with the basic properties of the smooth muscle in cerebral blood vessels. Even the mechanical response of these vessels to neurogenic transmitters and vasoactive substances *in vitro* is still not fully understood. Recently we found that γ-aminobutyric acid (GABA), a putative postsynaptic inhibitory neurotransmitter in the central nervous system, causes relaxation of dog cerebral arteries. Thus, the present experiments were undertaken to explore this effect and to investigate the possible mechanism by which GABA inhibits the vascular smooth muscle of dog cerebral arteries.

Methods Dogs of either sex weighing on the average 11 kg were anaesthetized with sodium pentobarbitone (45 mg/kg i.p.), bled through the common carotid arteries and their brains removed. The basilar artery, middle and posterior arteries were dissected from the brain and used in these experiments. The external diameter of the basilar arteries was approximately 0.9 mm (0.7-1.2 mm, $n = 30$) and of the middle and posterior cerebral arteries about 0.8 mm (0.65-1.0 mm, $n = 38$).

For measurement of their mechanical responses the arteries were prepared in helical strips approximately 1-1.5 mm in width and 15 mm in length and mounted vertically in an organ bath containing 20 ml of Krebs-Ringer solution of the following composition (mM): NaCl 154, KCl 5.4, $CaCl_2$ 2.2, $NaHCO_3$ 6, dextrose 11, in distilled, deionized water. The bath medium was maintained at 37°C, pH 7.4 and equilibrated before and during the experiment with a gas mixture of 95% O_2 and 5% CO_2. A resting tension of 0.5 g was initially applied and maintained throughout the experiment and the preparations were allowed to equilibrate for 90 min in the bathing medium before the experiments were commenced. During the equilibration period, the bath solution was changed every 20 minutes. The muscle tension changes were recorded isometrically through a force-displacement transducer (Nihon Koden) and displayed on a polygraph.

In some experiments helical strips of aorta from rabbits (2-3 kg), rings of thoracic aorta of dog, and strips of mesenteric artery and portal vein of both rabbit and dog were used as model peripheral blood vessels for comparison.

Results GABA at 10^{-7} to 10^{-5} M caused relaxation in 9 of 14 strips of basilar and 8 of 11 strips of middle cerebral arteries. The inhibitory effect of GABA was much greater in presence of active tension (5-HT, 10^{-8} M) than at resting tension (Figure 1). However, in most of the basilar and middle cerebral arteries, the response was observed only at high GABA concentration (Figure 1a, 1b). In 5 of 14 basilar strips and 4 of 11 middle cerebral arterial strips, a low concentration was sufficient to cause relaxation (Figure 1c). Even after application of a high concentration of GABA (10^{-5} M), the strips completely recovered their original tension after the relaxation. This inhibitory effect of GABA was only observed in cerebral arteries, but not in dog and rabbit aorta, mesenteric artery and portal vein. Treatment with glycine and glutamate (10^{-8}-10^{-5} M) had no effect on either middle cerebral or basilar arteries (Figure 1a, 1b). After pre-treatment with picotoxin (10^{-5} M) for several min, the relaxing effect of GABA was almost completely abolished (Figure 1d); however, pretreatment with phentolamine (10^{-6} M), propranolol (10^{-6} M), atropine (10^{-6} M), pentolinium (10^{-5} M), tetrodotoxin (10^{-7} M), reserpine (4 mg/kg i.m., 24 h earlier) and ouabain

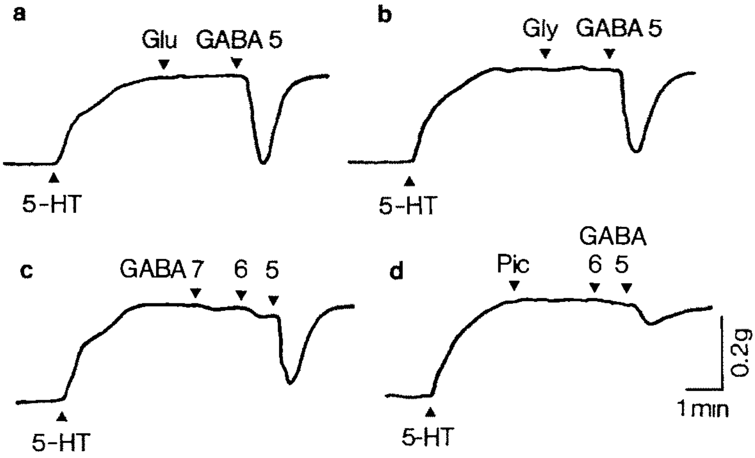

Figure 1 Relaxant effect of γ-aminobutyric acid (GABA), glutamate and glycine on the dog cerebral arteries. Relaxant effect was tested on the strips contracted by 5-hydroxytryptamine (5-HT) at 10^{-8} M. GABA 7 6 5 = 10^{-7} M, 10^{-6} M, and 10^{-5} M, glutamate (Glu) = 10^{-5} M; glycine (Gly) = 10^{-5} M; picrotoxin (Pic) = 10^{-7} M. (a) and (b) were from the same tissues; (c) and (d) were from the same tissues.

(5×10^{-8}-10^{-7} M) did not affect the response to GABA. Adenosine 5-triphosphate sodium (ATP) (10^{-5} M) and adenosine 5-monophosphate (10^{-5} M) caused similar relaxation of dog cerebral arteries but picrotoxin (10^{-5} M) failed to antagonize the effect.

Discussion GABA has a selective inhibitory effect on the dog cerebral arteries but not on the peripheral blood vessels of dog or rabbit. Other amino acids such as glycine and glutamate have no effect on the cerebral blood vessels. Since this inhibitory action of GABA was not blocked by adrenoceptor and ganglion blocking agents and by reserpine, atropine, and tetrodotoxin, the inhibitory effect of GABA is due to a direct action on the effector site rather than indirectly through the release of a chemical transmitter from the nerve endings. The antagonism of picrotoxin towards the response to GABA but not that induced by ATP or adenosine, plus the presence of GABA transaminase in cerebral arteries (Van Gelder, 1965), strongly suggest the existence of a specific GABA receptor in the vascular smooth muscle of the dog cerebral arteries.

This work was supported in part by the Nippon Tobacco and Salt Public Corporation and the Hawaii Heart Association. S.S. was visiting Professor at Kyoto University, School of Medicine during a sabbatical year of leave from the University of Hawaii. Reprint requests should be sent to Dr S. Shibata, University of Hawaii.

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(Received September 18, 1975)

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VOLUME 55, 1975

LONDON
MACMILLAN JOURNALS LIMITED
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British Journal of Pharmacology

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561 FUJIWARA, M., MURAMATSU, I. & SHIBATA, S. γ-Aminobutyric acid receptor on vascular smooth muscle of dog cerebral arteries.

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